

**COMPOUNDS AND METHODS FOR REGULATING  
BACTERIAL GROWTH AND PATHOGENESIS**

Related Application Information

[0001] This application is a continuation of U.S. Patent Application No. 10/300,818, filed November 19, 2002, which is a divisional of U.S. Patent Application No. 09/853,832, filed May 10, 2001, which claims priority to U.S. Provisional Application No. 60/203,000, filed May 10, 2000 and U.S. Provisional Application No. 60/254,398, filed December 7, 2000, all of which are herein incorporated by reference.

Statement of Government Interest

[0002] The U.S. Government has certain rights in this invention, which was made in part with funds from the National Science Foundation, Grant No. MCB-9506033.

Background of the Invention

Field of the Invention

[0003] This invention relates to bacterial diseases of humans and other mammals. In particular, the invention provides a novel signaling factor involved in regulating bacterial growth and pathogenesis, analogs and derivatives of the signaling factor, and methods for controlling bacterial growth and pathogenesis through use of such analogs and derivatives.

Background of the Invention

[0004] Intercellular cooperation confers a considerable advantage on multicellular organisms that was thought to be unavailable to unicellular organisms such as prokaryotes. Research in the last twenty years has revealed, however, that prokaryotes can communicate with each other in a way that modulates gene expression, and thereby can reap benefits that would otherwise be exclusive to eukaryotes. This ability was discovered in luminous marine bacteria such as *Vibrio fischeri* and *Vibrio harveyi*, which activate the expression of genes involved in light production only when their population density exceeds a critical value. This phenomenon, known as quorum-sensing, is now recognized as a general mechanism for gene

regulation in many Gram-negative bacteria, and it allows them to perform in unison such activities as bioluminescence, swarming, biofilm formation, production of proteolytic enzymes, synthesis of antibiotics, development of genetic competence, plasmid conjugal transfer, and sporulation.

[0005] Quorum-sensing bacteria fall into two classes, depending on how many density-sensing systems they have. Both classes synthesize, release, and respond to signaling molecules called autoinducers to control gene expression as a function of cell density. Bacteria in the larger class use acyl-homoserine lactone signals in a single density-sensing system, with one gene that encodes an autoinducer synthase, and another that encodes a transcriptional activator protein that mediates response to the autoinducer. These genes are homologous to *luxI* and *luxR* of *V. fischeri*, respectively (Bassler and Silverman, in *Two component Signal Transduction*, Hoch *et al.*, eds, Am. Soc. Microbiol. Washington D.C., pp 431-435, 1995).

[0006] Many bacteria that use the autoinducer-1 signaling factor associate with higher organisms, i.e., plants and animals, at some point during their lifecycles. For example, *Pseudomonas aeruginosa*, an opportunistic pathogen in humans with cystic fibrosis, regulates various virulence determinants with autoinducer-1. Other examples of autoinducer-1-producing bacteria include *Erwinia carotovora*, *Pseudomonas aureofaciens*, *Yersinia enterocolitica*, *Vibrio harveyi*, and *Agrobacterium tumefaciens*. *E. carotovora* infects certain plants and creates enzymes that degrade the plant's cell walls, resulting in what is called "soft rot disease." *Yersinia enterocolitica* causes gastrointestinal disease in humans and reportedly produces an autoinducer. *P. aureofaciens* synthesizes antibiotics under autoinducer control that block fungus growth in the roots.

[0007] Bacteria of the other class, exemplified by *V. harveyi*, have not one but two independent density-sensing systems. *V. harveyi* apparently uses the more species-specific Signaling System 1 for intra-species communication, and the less species-selective Signaling System 2 for inter-species communication (Bassler *et al.*, J. Bacteriol. 179: 4043-4045, 1997). Each system comprises a sensor-autoinducer pair; Signaling System 1 uses Sensor 1 and autoinducer-1 (AI-1), while Signaling System 2 uses Sensor 2 and autoinducer-2 (AI-2) (Bassler *et al.*, Mol. Microbiol. 13: 273-286, 1994). While autoinducer-1 is *N*-(3-

hydroxy butanoyl)-L-homoserine lactone (HSL) (see Bassler *et al.*, Mol. Microbiol. 9: 773-786, 1993), the structure of autoinducer-2 has not been established, nor have the gene(s) involved in its biosynthesis been identified.

[0008] Recent research indicates that quorum-sensing takes place not only among luminous marine bacteria, but also among pathogenic bacteria, where it regulates the production of virulence factors that are critical factors in bacterial pathogenesis. Thus, it would be an advance in the art to identify and characterize compounds with autoinducer-2 activity and the genes encoding the proteins required for production of the naturally-occurring autoinducer-2. Such an advance would provide a way to identify compounds useful for controlling pathogenic bacteria, a way to augment traditional antibiotic treatments, and a new target for the development of new antimicrobial agents.

#### Summary of the Invention

[0009] The applicants have now discovered that many bacteria, including some non-luminous pathogens, secrete a signaling molecule that mimics *V. harveyi* autoinducer-2 in its function and physical properties. Bacteria that produce the AI-2 signaling factor of the invention include *Vibrio harveyi*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Pseudomonas phosphoreum*, *Yersinia enterocolitica*, *Escherichia coli*, *Salmonella typhimurium*, *Haemophilus influenzae*, *Helicobacter pylori*, *Bacillus subtilis*, *Borrelia burgdorferi*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Yersinia pestis*, *Campylobacter jejuni*, *Deinococcus radiodurans*, *Mycobacterium tuberculosis*, *Enterococcus faecalis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes* and *Staphylococcus aureus*.

[0010] Free-living bacteria produce this novel signaling molecule only upon shifting to a colonizing, and therefore potentially pathogenic, existence in a host organism. Thus, in addition to stimulating luminescence genes in *V. harveyi*, the signaling molecule is expected to stimulate genes related to pathogenesis in bacteria that produce it. In addition to a purified molecule with autoinducer-2 signaling activity, the invention provides a synthetic form of the molecule and derivatives of it that regulate bacterial growth and pathogenesis.

[0011] In another aspect, there is provided a method for regulating the activity of an autoinducer-2 receptor comprising contacting an autoinducer-2 receptor with an AI-2 agonist or antagonist compound.

[0012] In another aspect, the invention provides a method of regulating autoinducer-2 activity by contacting a bacterial cell comprising autoinducer-2, or extract thereof, with a compound of structure I, II, III or IV.

[0013] In yet another aspect, the invention provides a method for regulating autoinducer-2 receptor activity by contacting an autoinducer-2 receptor with a compound of structure I, II, III or IV.

[0014] In another aspect, the invention provides a method for controlling bacterial growth or virulence by identifying a subject infected with an autoinducer-2-producing bacterium and administering to the subject a compound of structure I, II, III or IV.

[0015] In yet another aspect, the invention provides a method for inhibiting bacterial growth or virulence in a subject, by identifying a subject in which bacteria are producing autoinducer-2 and administering to the subject an inhibitor of an autoinducer-2 of the present invention.

[0016] In another aspect, the invention provides a method for identifying a compound that regulates the activity of autoinducer-2 by comparing the activity of autoinducer-2 obtained in the presence of the compound to that obtained in its absence.

[0017] In another aspect, the invention provides a method for identifying an autoinducer analog by contacting a cell, or cell extract, that produces a detectable amount of light in response to an autoinducer with the autoinducer analog and comparing the amount of light produced in the presence and the absence of the autoinducer analog.

[0018] In another aspect, the invention provides a method for identifying a compound that regulates the production or activity of autoinducer-2 by contacting with the compound a cell that produces autoinducer-2, and determining whether autoinducer-2 activity is present in the cell.

[0019] In another aspect, the invention provides a method for identifying a compound that affects binding of autoinducer-2 to an autoinducer-2 receptor by: (a) contacting autoinducer-2 and the autoinducer-2 receptor with the compound; (b) contacting

(a) with a cell, or cell extract, that produces light in response to autoinducer-2 binding to the autoinducer-2 receptor; and (c) measuring the effect of the compound on light production.

[0020] In yet another aspect, the invention provides a method for identifying a compound that affects autoinducer-2 binding to an autoinducer-2 receptor by: (a) contacting with the compound a complex formed between autoinducer-2 and the autoinducer-2 receptor to allow dissociation of the complex; (b) contacting (a) with a cell, or cell extract thereof, that produces light in response to binding of autoinducer-2 to the autoinducer-2 receptor; and (c) measuring the effect of the compound on light production.

[0021] In yet another aspect, the invention provides a method for regulating expression of a siderophore in a bacterial cell by contacting a cell capable of producing the siderophore with a compound of structure I, II, III or IV.

[0022] In another aspect, the invention provides a method for regulating exopolysaccharide production in a cell by contacting a cell capable of producing an exopolysaccharide with a compound of structure I, II, III or IV.

[0023] In another aspect, the invention provides a method for regulating bacterial colony morphology by contacting a bacterial colony with a compound of structure I, II, III or IV.

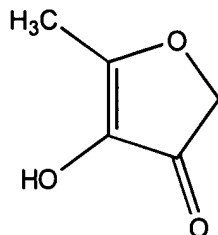
[0024] In another aspect, the invention provides a method for regulating bacterial biofilm formation by contacting a bacterium capable of biofilm formation with any combination of compounds set forth in structures I-IV.

[0025] In another aspect, the invention provides a method for producing autoinducer-2 by contacting S-adenosylhomocysteine (SAH) with a LuxS protein.

[0026] In another aspect, the invention further provides a method for producing autoinducer-2 by; a) contacting S-adenosylhomocysteine (SAH) with a 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase (pfs) protein to form S-ribosylhomocysteine; b) contacting the S-ribosylhomocysteine from a) with a LuxS protein to promote the conversion of S-ribosylhomocysteine to autoinducer-2.

[0027] In yet another aspect, the invention further provides a method for detecting an autoinducer-associated biomarker by: (a) contacting at least one cell with an autoinducer to promote induction of a biomarker; and (b) detecting the biomarker.

[0028] In another aspect, the invention provides a method for regulating bacterial cell growth or expression of a virulence factor comprising contacting a bacterial cell with an isolated autoinducer-2 analog comprising the structure:



[0029] The invention further provides a synergistic antibiotic composition comprising an antibiotic and an inhibitor of the quorum-sensing pathway of a microorganism.

[0030] The invention further provides a medical device comprising a synergistic antibiotic composition comprising an antibiotic and an inhibitor of the quorum-sensing pathway of a microorganism, as well as a method of treating infections in a warm-blooded animal caused by microorganisms possessing a quorum-sensing mechanism, comprising administering to the animal a therapeutically effective amount of the synergistic antibiotic composition.

[0031] The invention further provides a pharmaceutical composition comprising a synergistic antibiotic composition comprising an antibiotic and an inhibitor of a quorum-sensing pathway of a microorganism or a pharmaceutically acceptable salt thereof and one or more pharmaceutically acceptable carriers, adjuvants or vehicles.

[0032] The invention also provides a method of treating infections in a warm-blooded animal caused by microorganisms possessing a quorum-sensing mechanism that comprises administering to the animal a therapeutically effective amount of the synergistic antibiotic composition comprising an antibiotic and an inhibitor of the quorum-sensing pathway of a microorganism.

[0033] The invention further provides a method for inhibiting biofilm formation by contacting a bacterium capable of biofilm formation with a compound having the structure set forth in structure III or structure IV.

[0034] The invention further provides a medical device comprising at least one antimicrobial compound having the structure set forth in structure III or structure IV, where

the device is supplemented with the compound and the compound is present in a concentration sufficient to provide a localized antimicrobial effect.

[0035] The invention further provides a medical device comprising at least one synergistic antibiotic composition of an antibiotic and an inhibitor of the quorum-sensing pathway of a microorganism, where the composition is present in a concentration sufficient to provide a localized antimicrobial effect.

[0036] The invention further provides a medical device comprising at least one pharmaceutical composition comprising a synergistic antibiotic composition comprising an antibiotic and an inhibitor of a quorum-sensing pathway of a microorganism or a pharmaceutically-acceptable salt thereof and one or more pharmaceutically acceptable carriers, adjuvants or vehicles, wherein the composition is present in a concentration sufficient to provide a localized anti-microbial effect.

[0037] Additional features and advantages of the present invention will be better understood by reference to the drawings, detailed description and examples that follow.

#### Brief Description of the Drawings

[0038] **Figure 1.** Signaling substance from *E. coli* AB1157 and *S. typhimurium* LT2 cell-free culture fluids that induces luminescence in *V. harveyi*. The responses of *V. harveyi* reporter strains BB170 (Sensor 1<sup>-</sup>, Sensor 2<sup>+</sup>) (Figure 1A), and BB886 (Sensor 1<sup>+</sup>, Sensor 2<sup>-</sup>) (Figure 1B) to signaling substances present in cell-free culture fluids from *E. coli*, *S. typhimurium* and *V. harveyi* strains are shown. A bright culture of each reporter strain was diluted 1:5000 into fresh medium, and the light production per cell was then measured during the growth of the diluted culture. Cell-free culture fluids or sterile growth medium were added at a final concentration of 10% (v/v) at the start of the experiment. The data for the 5-hour time point are shown and are presented as the percent of the activity obtained when *V. harveyi* cell-free spent culture fluids are added. Abbreviations used for the different strains are: V.h; *Vibrio harveyi*, S.t; *Salmonella typhimurium*, and E.c; *Escherichia coli*.

[0039] **Figure 2.** Secretion of the autoinducer-2 signaling molecule by viable *E. coli* and *S. typhimurium*. The response of the *V. harveyi* reporter strain BB170 (Sensor 1<sup>-</sup>, Sensor 2<sup>+</sup>) to a signaling substance produced and secreted by *E. coli* AB1157 and *S.*

*typhimurium* LT2 but not *E. coli* DH5a is shown. *V. harveyi* reporter strain BB170 was diluted 1:5000 in AB medium and light output per cell was monitored during growth. At the start of the experiment, either  $1 \times 10^6$  *E. coli* AB1157, *S. typhimurium* LT2 or *E. coli* DH5a washed and resuspended viable cells (left-hand, white bars) or UV-killed cells (right-hand, black bars) was added. The data are presented as the fold-activation above the endogenous level of luminescence expressed by *V. harveyi* BB170 at the 5-hour time point. Abbreviations used for the different strains are: S.t; *Salmonella typhimurium*, and E.c; *Escherichia coli*.

**[0040]** **Figure 3.** Effect of glucose depletion on the production and degradation of the autoinducer-2 signaling activity by *S. typhimurium* LT2. *S. typhimurium* LT2 was grown in LB medium containing either 0.1% glucose (Figure 3A) or 0.5% glucose (Figure 3B). At the specified times cell-free culture fluids were prepared and assayed for signaling activity in the luminescence stimulation assay (Bars), and the concentration of glucose remaining (circles). The cell number was determined at each time by diluting and plating the *S. typhimurium* LT2 on LB medium and counting colonies the next day (squares). The signaling activity is presented as the percent of the activity obtained when *V. harveyi* cell-free spent culture fluids are added. These data correspond to the 5 h time point in the luminescence stimulation assay. The glucose concentration is shown as % glucose remaining. Cell number is cells/ml  $\times 10^{-9}$ . The symbol \\\ indicates that the time axis is not drawn to scale after 8 h.

**[0041]** **Figure 4.** Response curve of *V. harveyi* to AI-2 produced by *V. harveyi* and *S. typhimurium*. The *V. harveyi* reporter strain BB170 (Sensor 1<sup>-</sup>, Sensor 2<sup>+</sup>) was tested for its response to the addition of exogenous AI-2 made by *V. harveyi* strain BB152 (AI-1<sup>-</sup>, AI-2<sup>+</sup>) and to that made by *S. typhimurium* LT2. A bright culture of the reporter strain was diluted 1:5000 and either 10% (v/v) growth medium (closed circles), cell-free culture fluid from *V. harveyi* BB152 grown overnight in AB (open circles), or cell-free culture fluid from *S. typhimurium* LT2 grown for 6 h on LB + 0.5% glucose (closed squares) was added at the start of the experiment. RLU denotes relative light units and is defined as (counts min<sup>-1</sup>  $\times 10^{-3}$ )/(colony-forming units ml<sup>-1</sup>).

**[0042]** **Figure 5.** Conditions affecting autoinducer production in *S. typhimurium*. *S. typhimurium* LT2 was subjected to a variety of treatments after which cell-free culture fluids or osmotic shock fluids were prepared. These preparations were added to a diluted



culture of the *V. harveyi* AI-2 reporter strain BB170 at 10% (v/v) and light output was measured thereafter. Fold activation is the level of light produced by the reporter following addition of the specified *S. typhimurium* preparation divided by the light output of the reporter when growth medium alone was added. The bars in Figure 5A represent cell-free fluids prepared from *S. typhimurium* after the following treatments: **LB 6h**; 6 h growth in LB at 30°C, **LB+Glc 6h**; 6 h growth in LB + 0.5% glucose at 30°C, **LB+Glc 24h**; 24 h growth in LB + 0.5% glucose at 30°C. In all the experiments presented in Figure 5B, the *S. typhimurium* were pre-grown at 30°C for 6 h in LB containing 0.5% glucose, then pelleted and resuspended for 2 h under the following conditions: **LB**; in LB at 30°C, **LB+Glc**; in LB + 0.5% glucose at 30°C, **LB pH 5**; in LB at pH 5.0 at 30°C, **0.4 M NaCl**; in 0.4 M NaCl at 30°C, **0.1M NaCl**; in 0.1M NaCl at 30°C, and **Heat Shock 43°**; in LB + 0.5% glucose at 43°C. After these two-hour treatments, cell-free fluids were prepared from each sample and assayed.

[0043] **Figure 6.** *S. typhimurium* signaling activity in limiting and non-limiting concentrations of glucose. *S. typhimurium* LT2 was grown in LB in the presence of limiting (0.1%) and non-limiting (1.0%) concentrations of glucose. The activity present in the cell-free culture fluids (black bars) was assayed at the times indicated and normalized to that produced by  $1 \times 10^9$  cells. The increase in signaling activity measured in the 0.4 M NaCl osmotic shock fluids prepared from the same cells is shown as the white bars on top of the black bars. These data are also normalized for  $1 \times 10^9$  cells. The signaling activity for limiting glucose is shown in Figures 6A, 6C, and 6E, and that for non-limiting glucose is shown in Figures 6B, 6D, and 6F. Figures 6A and 6B also show the percent glucose remaining (triangles), Figures 6C and 6D show the cell number (squares), and Panels E and F show the pH (circles) at each time point.

[0044] **Figure 7.** Effects of glucose and pH on signal production by *S. typhimurium*. The quorum-sensing signal released by *S. typhimurium* LT2 was measured when the cells were grown in LB medium containing 0.5% glucose at pH 7.2 (Figure 7A, bars), and when the cells were grown in LB at pH 5.0 without an added carbon source (Figure 7B, bars). The level of signal present in cell free culture fluids (black bars) and in 0.4 M NaCl osmotic shock fluids was measured (white bars on top of black bars) at the time points

indicated. In each panel, the circles represent the pH of the medium, and the squares show the cell number at the different time points.

[0045] **Figure 8.** High osmolarity induces signal release and low osmolarity induces signal degradation in *S. typhimurium* LT2. The quorum-sensing signal released by *S. typhimurium* LT2 resuspended in 0.4 M NaCl and in 0.1M NaCl was measured in the presence and absence of protein synthesis. *S. typhimurium* LT2 was pre-grown in LB containing 0.5% glucose for 6 h. The cells were harvested and resuspended in 0.4 M NaCl (Figure 8A) or 0.1 M NaCl (Figure 8B) in the presence or absence of 30 g/ml Cm for the time periods indicated. In each panel, the open symbols represent the activity measured in the absence of Cm and the closed symbols represent the activity measured in the presence of Cm.

[0046]. **Figure 9.** The *luxS* and *ygaG* genes from *V. harveyi* and *E. coli* MG1655. Figure 9A shows a restriction map of the *V. harveyi luxS<sub>V.h.</sub>* chromosomal region which was defined by Tn5 insertion. The sites of Tn5 insertions that disrupted the AI-2 production function and one control Tn5 insertion outside of the *luxS<sub>V.h.</sub>* locus are shown (triangles). Figure 9B depicts the *ygaG* region in the *E. coli* MG1655 chromosome. This ORF is flanked by the *emrB* and *gshA* genes. The direction of transcription of each gene is indicated by the horizontal arrows. The corresponding position of the MudJ insertion that eliminated AI-2 production in *S. typhimurium* LT2 is shown by a vertical arrow. H, R, P, and B denote *HindIII*, *EcoRI*, *PstI* and *BamHI* restriction sites, respectively.

[0047] **Figure 10** shows autoinducer production phenotypes of *V. harveyi* and *S. typhimurium* strains. Cell-free culture fluids from *V. harveyi* and *S. typhimurium* strains were prepared and tested for AI-2 activity in the *V. harveyi* BB170 bioassay. Figure 10A: AI-2 production phenotypes of the wild type *V. harveyi* strain MM28 which contains a Tn5 insertion outside of *luxS<sub>V.h.</sub>* (denoted WT) and the *luxS<sub>V.h.</sub>::Tn5* mutant strain MM30 (denoted *luxS*). Figure 10B: AI-2 production phenotypes of wild type *S. typhimurium* LT2 (denoted WT) and the *ygaG::MudJ* insertion mutant strain CS132 (denoted *ygaG*). Activity is reported as fold-induction of luminescence expression of the *V. harveyi* BB170 reporter strain over that when sterile medium was added.

[0048] **Figure 11** is a graph showing complementation of AI-2 production in *S. typhimurium* CS132 and *E. coli* DH5 $\alpha$ . Cell-free culture fluids from *E. coli* and *S.*

*typhimurium* strains were tested for AI-2 activity in the bioassay. The activity present in these fluids was compared to that produced by wild type *V. harveyi* BB120. In the figure, the level of BB120 activity was normalized to 100%. Figure 11A: AI-2 activity in cell-free fluids from wild type *V. harveyi* BB120, *E. coli* O157:H7, and *S. typhimurium* LT2. Figure 11B: Complementation of *S. typhimurium* CS132 (*ygaG*::MudJ) and Figure 11C: Complementation of *E. coli* DH5 $\alpha$ . In Panel B and C, the *in trans* AI-2 production genes are the following: vector control (denoted: none), *E. coli* O157:H7 *ygaG*; and *V. harveyi* BB120 *luxS<sub>V.h.</sub>* *E. coli* and *V. harveyi* are abbreviated *E.c.* and *V.h.*, respectively.

[0049] **Figure 12** shows the alignment of LuxS and YgaG protein sequences. The translated protein sequences for the AI-2 production family of proteins are shown. We determined the sequences for the *luxS<sub>V.h.</sub>* gene from *V. harveyi* BB120 (SEQ ID NO: 10), and the *ygaG* genes (re-named herein as *luxS<sub>E.c.</sub>* from *E. coli* MG1655 (SEQ ID NO: 25), *E. coli* O157:H7 (SEQ ID NO: 11), and *E. coli* DH5 $\alpha$  (SEQ ID NO: 26). The *S. typhimurium* LT2 *ygaG* (re-named herein *luxS<sub>S.t.</sub>* partial sequence came from the *S. typhimurium* database. Amino acid residues that are not identical to the LuxS<sub>V.h.</sub> protein are underlined and not in bold font. The site of the frame shift mutation in the *E. coli* DH5 $\alpha$  DNA sequence is denoted by an “\*.” The 20 altered amino acid residues that are translated following the frame shift are enclosed by the box.

[0050] **Figure 13** provides a diagram of the hybrid quorum-sensing circuit of *Vibrio harveyi*. The AI-1 and AI-2 circuits are independently stimulated but integrate their signals for light expression. Each pathway, however, is also independently competent to generate light. This allows for reciprocal mutations in the LuxN or LuxQ sensors to be used to construct a reporter specific for AI-2 or AI-1, respectively.

[0051] **Figure 14** is a graph representing response phenotypes of *V. harveyi* wild type and lux regulatory mutants. At the first time point, cell-free culture fluids (10%), or nothing (N.A.) was added. Wild type, cell-free culture fluid (AI-1 + AI2); LuxS<sup>-</sup> cell-free culture fluid (AI-1); LuxM<sup>-</sup> cell-free culture fluid (AI-2). Relative light units are defined as cpm x 10<sup>3</sup>/CFU/ml.

[0052] **Figure 15** shows a diagram of the biosynthetic pathway of autoinducer-2 (AI-2).

[0053] **Figure 16** shows bar graphs indicating the result of luminescence screening assays of AI-2 analogs. Figure 16 panels A and B show the results of a 6 hour assay using compounds 6, 9, 15 and 18 (panel A) and compounds 3, 4, 7 and 13 (panel B) on test strain BB170. Figure 16 panels C and D show the effect of the same compounds on control strain JAF78.

[0054] **Figure 17** shows examples of structures of analog compounds that inhibit the activity of AI-2 in the *V. harveyi* luminescence assay. The structures of these compounds indicate that the C2 and C3 positions are important to the inhibitory activity of the compounds.

[0055] **Figure 18** is a bar graph showing the effect of compounds QXP009, QXP010, QXP015 and QXP018 on AI-2 activity in the *V. harveyi* luminescence assay.

[0056] **Figure 19** shows a list of compounds and their effect on AI-2 activity in the *V. harveyi* luminescence assay.

[0057] **Figure 20** is a photograph showing  $\sigma^{54}$  and LuxO regulation of colony morphology in *V. harveyi*. The smooth and rugose colony morphologies of different *V. harveyi* strains are shown in the photographs. Each *V. harveyi* strain was grown in LM broth overnight at 30°C. The strains were streaked onto LM plates, grown for 24 hr at 30°C and photographed. The strain denotations are the following: wt, BB120; luxO D47E, JAF548; rpoN::Cmr, BNL240; and luxO D47E, rpoN::Cmr, BNL244. Both BNL240 and BNL244 were supplemented with 1 mM L-glutamine in broth and on plates.

[0058] **Figure 21** shows the effect of compound QXP031 on bioluminescence intensity in *V. harveyi* tester strain. The results are normalized to cell count and control strain. Results demonstrate up to 15000 fold specific decrease in bioluminescence as a result of AI-2 inhibition at various compound QXP031 concentrations.

[0059] **Figure 22** shows a CAMP assay indicating inhibition of CAMP expression by compound QXP031. The horizontal streak is *S. aureus* 25923 and the vertical streaks are *S. pyogenes* 19615. Filter disks containing either DMSO no drug control or compound QXP031 were applied to plates and incubated anaerobically overnight at 37°C. No compound control results in a positive CAMP score as indicated by the arrow shaped region

of complete beta-hemolysis. Application of QXP031 results in a complete inhibition of hemolysis.

[0060] **Figure 23** shows inhibition proteolysis of milk protein by compound QXP031 ((R)-4-acetoxy-cyclopent-2-eneone). Skim milk containing plates were overlaid with top agar containing *S. pyogenes* 19615. Filter disks containing either DMSO no compound control or compound QXP031 were applied to plates and incubated anaerobically overnight at 37°C. As shown in Figure 23, the entire plate clears due to *S. pyogenes* proteolytic activity except for the zone surrounding the QXP031 containing disc where proteolysis was inhibited.

[0061] **Figure 24** shows induction of bioluminescence by synthetic 4-hydroxy-5-methyl-2h-furan-3-one. The MM32 indicator strain (LuxS-, LuxN-) of *V. harveyi* was incubated with various doses of AI-2 and the bioluminescence signal measured at various time points during a 6-hour incubation.

[0062] **Figure 25** shows biofilm formation for wild-type *V. harveyi* (strain BB120), strain MM32 (LuxN-, LuxS-) and *V. harveyi* indicator strain BB170 (LuxN-). The strains were tested for biofilm formation using either AB media with supplemented with glucose (ABG; Panel A) or LB media supplemented with glucose (LBG; Panel B). Absorbance reading at 600 nm (A600) were used to measure the amounts of crystal violet adhered to the biofilm.

[0063] **Figure 26** shows *V. harveyi* biofilm formation in response to increasing amounts of 4-hydroxy-5-methyl-2h-furan-3-one. The final concentration of AI-2 in each assay is indicated.

[0064] **Figure 27** shows *Pseudomonas aeruginosa* biofilm formation in response to AI-2. *Pseudomonas aeruginosa* (ATCC 27853) were tested for biofilm formation using AB media supplemented with glucose (LBG). Absorbance reading at 600 nm (A600) were used to measure the amounts of crystal violet adhered to the biofilm. Doses in the legend refer to the final concentration of AI-2 in the assay.

[0065] **Figure 28** shows a BB170 assay measuring the effect of QXP-048 (2-methoxy-2,4-diphenyl-3(2H)furanone) on the bioluminescence signal emanating from

endogenously produced autoinducer-2. QXP-048 was added at the outset of the assay at a final concentration of 25 µg/ml.

[0066] **Figure 29** shows a BB170 Assay measuring the effect of QXP-048 on signal from an exogenous autoinducer-2 signal. Exogenous synthetic autoinducer-2 was added at the outset of the assay at a final concentration of 25 µg/ml. QXP-048 was added at the outset of the assay at a final concentration of 25 µg/ml. **Figure 30** shows an MM32 assay further identifying the inhibitory capability of compound QXP-048. Exogeneous synthetic autoinducer-2 was added at the outset of the assay to a final concentration of 25 µg/ml. QXP-048 was added at the outset of the assay at a final concentration of 25 µg/ml.

[0067] **Figure 31** shows inhibition of bioluminescence by compound QXP-035. Various amounts of QXP-035 were used in BB170-based AI-2-dependent bioluminescence assay (indicated in micrograms/milliliter in the figure legend) and the effects on bioluminescence monitored over time.

[0068] **Figure 32** shows the effect of compound QXP-035 (2-ethyl-4-hydroxy-5-methyl-3(2H)-furanone) on cell growth. *Streptococcus pyogenes* ATCC 19615 was grown in the presence of either 2.4 µg/ml or 2.5 mg/ml of compound QXP-035. Cell growth was monitored by taking periodic measurements of absorbance at 600 nm.

[0069] **Figure 33** are examples of inhibitory (Column A) and stimulatory (Columns B and C) compounds for the AI-2 receptor.

[0070] **Figure 34** are examples of stimulatory compounds for the AI-2 receptor.

### Detailed Description of the Invention

#### Definitions

[0071] “Lower alkyl” refers to alkyl groups have from one to about ten carbon atoms.

[0072] “Isomer” means stereoisomers, and includes enantiomers and diastereomers.

[0073] “Purified from a native source” includes autoinducer-2 analogs that have been manufactured by an organism, and includes isolating an autoinducer-2 analog from the

culture medium or cytoplasm of bacteria such as *S. typhimurium* through use of conventional purification techniques.

[0074] “Inhibiting infectivity” includes methods of affecting the ability of a pathogenic organism to initially infect or further infect a subject that would benefit from such treatment.

[0075] “Biomarker” refers to any bacterial cell component that is identifiable by known microscopic, histological, or molecular biological techniques. Such a biomarker can be, for example, a molecule present on a cell surface, a protein, a nucleic acid, a phosphorylation event, or any molecular or morphological characteristic of a bacterial cell that changes when the bacterium is in the presence of an autoinducer.

[0076] A “probe” can be a nucleic acid, protein, small molecule, or antibody useful for detecting a bacterial biomarker present in a sample.

[0077] As used herein, the term “medical device” means a device having surfaces that contact tissue, blood, or other bodily fluids in the course of their operation. This definition includes within its scope, for example, surgical implants, surgical sutures, wound dressings, extracorporeal devices for use in surgery such as blood oxygenators, blood pumps, blood sensors, tubing used to carry blood and the like which contact blood which is then returned to the subject. The definition includes within its scope endoprostheses implanted in blood contact in a human or animal body such as vascular grafts, stents, pacemaker leads, heart valves, and the like that are implanted in blood vessels or in the heart. The definition also includes within its scope devices for temporary intravascular use such as catheters, guide wires, and the like which are placed into the blood vessels or the heart for purposes of monitoring or repair.

[0078] A “compound” can be any agent, composition, or molecule that affects the activity of AI-2 or affects the activity of a protein that binds with AI-2. For example, a compound of the invention can be a nucleic acid, a protein, an analog of AI-2 or small molecule. A “compound” includes molecules that regulate the autoinducer-2 receptors, such as LuxP or LuxQ. For example, a compound of Structure I, II, III or IV can regulate the activity of a LuxP protein, a LuxQ protein, a LuxP-LuxQ complex, a LuxP-AI-2 complex, or a LuxP-LuxQ-AI-2 complex, such that bacterial growth or the expression of an extracellular

virulence factor is regulated. Such compounds include inhibitors that interact directly with AI-2 such that AI-2 is prevented from acting as a sensor for quorum-sensing Signaling System-2. For example, a compound of the invention could interact directly with 4,5-dihydroxy-2-cyclopenten-1-one or 4-hydroxy-5-methyl-2H-furan-3-one.

[0079] AI-2 “inhibitor” refers to molecules that interfere with the ability of the autoinducer to act as a signal for luminescence, bacterial growth or pathogenesis, and includes molecules that degrade or bind to AI-2. Inhibitors also include those compounds that regulate AI-2 activity by interacting with those proteins normally associated with bacterial growth.

[0080] The “activity” of AI-2 encompasses any aspect of AI-2’s ability to act as a signaling factor in bacterial quorum sensing, growth regulation, and pathogenesis.

[0081] “Autoinducer-2 analog” means any compound with at least 10% of the autoinducer-2 activity of any stereoisomer of 4-hydroxy-5-methyl-2H-furan-3-one, and includes the naturally-occurring autoinducer-2.

[0082] “Signaling factor,” “signaling molecule,” “autoinducer,” and more specifically, “autoinducer-2,” or “AI-2” all refer to the novel signaling factor of the present invention. The terms “autoinducer-2” and “AI-2” refer specifically to the signaling factor involved in quorum sensing system 2. The terms “signaling factor” or “signaling molecule,” “autoinducer” or “AI-2-like molecule” refer generally to the signaling factors of the present invention, of which AI-2 is an example.

[0083] “Isolated nucleic acid”, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous (in the 5’ and 3’ directions) in the naturally occurring genome of the organism from which it was derived. For example, the “isolated nucleic acid” may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a procaryote or eucaryote. An “isolated nucleic acid molecule” may also comprise a cDNA molecule.

[0084] “Isolated nucleic acid” primarily refers to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from RNA molecules with which it would be



associated in its natural state (*i.e.*, in cells or tissues), such that it exists in a “substantially pure” form (the term “substantially pure” is defined below).

[0085] “Isolated protein” or “isolated and purified protein” refers primarily to a protein produced by expression of an isolated nucleic acid molecule of the invention. Alternatively, this term may refer to a protein that has been sufficiently separated from other proteins with which it would naturally be associated, to exist in “substantially pure” form.

[0086] “Substantially pure” refers to a preparation comprising at least 50-60% by weight the factor of interest (e.g., pathogenesis signaling factor, nucleic acid, oligonucleotide, or protein). More preferably, the preparation comprises at least 75% by weight, and most preferably 90-99% by weight, the factor of interest. Purity is measured by methods appropriate for the factor of interest (e.g., chromatographic methods, agarose or polyacrylamide gel electrophoresis, HPLC analysis, and the like).

[0087] “Immunologically specific” refers to antibodies that bind to one or more epitopes of a protein of interest, but that do not substantially recognize and bind other molecules in a sample containing a mixed population of antigenic biological molecules.

[0088] “Specifically hybridizing” refers to the association between two single-stranded nucleotide molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed “substantially complementary”). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA or RNA molecule of the invention, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence.

[0089] “Promoter region” refers to the transcriptional regulatory regions of a gene, which may be found at the 5’ or 3’ side of the coding region, or within the coding region, or within introns.

[0090] “Selectable marker gene” refers to a gene encoding a product that, when expressed, confers a selectable phenotype such as antibiotic resistance on a transformed cell.

[0091] “Reporter gene” refers to a gene that encodes a product that is easily detectable by standard methods, either directly or indirectly.

[0092] “Operably linked” means that the regulatory sequences necessary for expression of the coding sequence are placed in the DNA molecule in the appropriate positions relative to the coding sequence to enable expression of the coding sequence. This same definition is sometimes applied to the arrangement of transcription units and other regulatory elements (e.g., enhancers or translation regulatory sequences) in an expression vector.

[0093] “Autoinducer-associated bacterial biomarker” refers to any bacterial cell component that an autoinducer regulates, modifies, enhances, inhibits, or induces.

[0094] The term “regulate” has its usual meaning, but also encompasses the meanings of the words enhance, inhibit, and mimic. In addition, as used herein, the term “expression” when used in connection with a gene such as LuxP, LuxQ or a gene encoding a virulence factor, has its usual meaning, but also encompasses the transcription of the gene, the longevity of functional mRNA transcribed from the gene, the translation of that mRNA, and the activity of the gene product. “Regulating” encompasses inhibition or activation of the autoinducer-2 signaling pathway.

[0095] “Antibody” includes intact molecules of polyclonal or monoclonal antibodies, as well as fragments thereof, such as Fab and F(ab')<sub>2</sub>. For example, monoclonal antibodies are made from antigen containing fragments of a protein by methods well known to those skilled in the art (Kohler et al., Nature, 256:495, 1975).

[0096] “Substantially the same” refers to nucleic acid or amino acid sequences having sequence variation that do not materially affect the nature of the protein (i.e., the structural characteristics and/or biological activity of the protein). With particular reference to nucleic acid sequences, the term “substantially the same” refers to the coding region and to conserved sequences governing expression, and refers primarily to degenerate codons encoding the same amino acid, or alternate codons encoding conservative substitute amino acids in the encoded polypeptide. With reference to amino acid sequences, the term “substantially the same” refers generally to conservative substitutions and/or variations in regions of the polypeptide not involved in determination of structure or function. The terms “percent identity” and “percent similarity” are also used herein in comparisons among amino acid sequences. These terms are defined as in the UWGCG sequence analysis program

(Devereaux et al., Nucl. Acids Res. 12: 387-397, 1984), available from the University of Wisconsin, and the parameters used by that program are the parameters to be used herein to compare sequence identity and similarity.

[0097] "Antibiotic" includes bactericidal, as well as bacteristatic agents, and includes sulfonamides, anti-urinary tract agents,  $\beta$ -lactam antibiotics, cephalosporins, clavulanic acid derivatives, aminoglycosides, tetracyclines and related antibacterial agents, macrolides, anti-tuberculosis drugs, anti-*Mycobacterium avium* agents, anti-leprosy agents, antifungal agents and antiviral agents.

[0098] "Sulfonamides" includes sulfanilamide, sulfamethoxazole, sulfacetamide, sulfadiazine, sulfisoxazole, para-aminobenzoic acid and the like, as well as trimethoprim-sulfamethoxazole.

[0099] "Quinolones" means nalidixic acid, cinoxacin, norfloxacin, ciprofloxacin, ofloxacin, sparfloxacin, lomefloxacin, fleroxacin, pefloxacin, amifloxacin and the like.

[0100] "Anti-urinary tract infectives" means methenamine, nitrofurantoin and the like.

[0101] By " $\beta$ -lactam antibiotics" we mean penicillins, cephalosporins and the like. By penicillins, examples are penicillin G, penicillin V, methicillin, oxacillin, cloxacillin, dicloxacillin, nafcillin, ampicillin, amoxicillin, carbenicillin, carbenicillin indanyl, ticarcillin, mezlocillin, piperacillin, bacampicillin and the like.

[0102] "Cephalosporins" means compounds such as cephalothin, cefazolin, cephalixin, cefadroxil, cefamandole, cefoxitin, cefaclor, cefuroxime, cefuroxime axetil, loracarbef, cefonicid, cefotetan, ceforanide, cefotaxime, cefpodoxime proxetil, ceftizoxime, ceftriaxone, cefoperazone, ceftazidime, cefepime, moxalactam and the like. By carbapenems we mean beta-lactam antibiotics such as imipenem, meropenem, aztreonam (imipenem-cilastatin).

[0103] "Clavulanic acid derivatives" means combinations of amoxicillin and clavulanic acid and ticarcillin and clavulanic acid.

[0104] "Aminoglycosides" means streptomycin, gentamicin, tobramycin, amikacin, netilmicin, kanamycin, neomycin and the like.

[0105] "Tetracyclines" means chlortetracycline, oxytetracycline, demeclocycline, methacycline, doxycycline, minocycline and the like.

[0106] "Related antibacterial agents" means chloramphenicol, clindamycin, spectinomycin, polymyxin B, colistin, vancomycin, bacitracin, RP 59500, glycyclcyclines and the like.

[0107] "Macrolides" means erythromycin, clarithromycin, azithromycin and the like.

[0108] "Anti-tuberculosis drugs" means isoniazid, rifampin, ethambutol, streptomycin, pyrazinamide, ethionamide, aminosalicylic acid, cycloserine, capreomycin and the like.

[0109] By drugs for the "anti-Mycobacterium avium agents" we mean rifabutin, macrolides, quinolones, clofazimine, amikacin and the like.

[0110] "Anti-leprosy agents" means sulfones such as dapsone and sulfoxone sodium, rifampin, clofazimine, thalidomide and ethionamide.

[0111] "Antifungal agents" means systemic antifungal agents such as amphotericin B, flucytosine, imidazoles and triazoles, ketoconazole, miconazole, itraconazole, fluconazole and the like. This category also includes griseofulvin. We also mean topical antifungal agents such as clotrimazole, econazole, miconazole, terconazole, butoconazole, ciclopirox olamine, haloprogin, tolnaftate, naftifine, terbinafine as well as nystatin, amphotericin B, undecylenic acid, benzoic acid, salicylic acid, propionic acid, caprylic acid and potassium iodide.

[0112] "Antiviral agents" means anti-herpes virus agents such as acyclovir, famciclovir, foscarnet, ganciclovir, idoxuridine, sorivudine, trifluridine, valacyclovir, vidarabine, penciclovir as well as newer agents such as anti HIV-1 such as lamivudine, FTC, adefovir (PMEA), nevirapine, delavirdine, zalcitabine, zidovudine, and the like; anti-hepatitis B virus agents such as lamivudine, famciclovir and fialuridine; anti-herpes viruses such as cidofovir (HPMPC) and lobucavir; anti papillomavirus agents such as afovirsen; anti rhinovirus agents such as sICAM-1, pirodavir, and the like; anti-influenza virus drugs such as GG167; anti-retroviral agents such as didanosine, stavudine, zalcitabine, zidovudine and the

like as well as other antiviral agents such as amantadine, interferon alpha, ribavirin and rimantadine.

[0113] In accordance with the present invention, the applicants have identified an analog of an extracellular signaling factor produced by several strains of pathogenic bacteria, including *Salmonella typhimurium* and *Escherichia coli*, that affects the pathogenesis or virulence of these bacteria. They have also identified and cloned genes involved in the biosynthesis of the naturally-occurring signaling factor. The identification of an autoinducer-2 analog and the cloning the genes that encode proteins involved in biosynthesis of the naturally-occurring autoinducer-2 open a new avenue for drug design aimed at affecting quorum sensing in bacteria. Drugs designed to interfere with quorum sensing constitute a new class of antibiotics. The invention further provides methods for detecting an autoinducer and methods for producing *in vitro* compounds with autoinducer-2 activity.

#### Preparation of the Factor with AI-2 Signaling Activity

[0114] Initial strategies for purifying the signaling molecule of the invention resulted in a partially purified preparation comprising the molecule with a specific signaling activity estimated at about 0.1 - 1.0 mg of the partially purified material stimulating a 1,000-fold increase in luminescence, as measured in the *V. harveyi* bioassay. The signaling activity does not extract quantitatively into organic solvents and it does not bind to either a cation or an anion exchange column. The molecule is a small (less than 1,000 kDa), polar but uncharged organic factor. The activity is acid stable and base labile, and it is heat resistant to 80°C but not 100°C. These features of the signaling molecule make it clear that the molecule is different from any previously described autoinducer.

[0115] The signaling factor of the present invention may be purified from its natural sources, *i.e.*, the bacteria that produce it. Alterations in the culture medium, *e.g.*, by addition of glucose or another sugar, an increase in the osmolarity, and/or decreases in pH, can increase production of the signaling molecule in *Salmonella* and other enteric bacteria and have enabled purification of the signaling molecule to near-homogeneity. Thus, the molecule has now been highly purified from culture fluids of enteric bacteria (*e.g.*, *E. coli*, *S. typhimurium*) through use of the following protocol:

[0116] Grow a culture of the signal producing enteric bacterium overnight in LB containing 0.5% glucose or another sugar (37°C, with aeration). Inoculate fresh LB containing glucose or another sugar at 0.5% with the overnight culture, at a 1:100 dilution. Grow the diluted culture to mid-exponential phase (3.5 h, 37°C, with aeration).

[0117] Pellet the cells (10,000 rpm, 10 min, 4°C). Discard the culture medium. Resuspend the cells and wash in 1/10th the original volume of low osmolarity NaCl solution (0.1M NaCl in water).

[0118] Pellet the cells again (10,000 rpm, 10 min, 4°C). Discard the low osmolarity culture fluid. Resuspend the cells in 1/10th the original volume of high osmolarity NaCl solution (0.4 M NaCl in water). Incubate the suspension at 37°C for 2 h with aeration. During this time, increased production and secretion of the signaling molecule occurs.

[0119] Pellet the cells (10,000 rpm, 10 min, 4°C). Collect the supernatant containing the secreted signaling molecule, and filter the supernatant through a 0.2 M bacterial filter to remove any remaining cells.

[0120] Evaporate the aqueous filtrate using a rotary evaporator at 30°C. Extract the dried filtrate in 1/10th the original volume of chloroform:methanol (70:30).

[0121] Evaporate the organic extract using a rotary evaporator at room temperature. Re-dissolve the dried extract in methanol at 1/100th of the original volume.

[0122] Subject the partially purified signal to high performance liquid chromatography (HPLC), using a preparative reverse phase C18 column. Elute the molecule with a linear gradient of 0-100% acetonitrile in water at 5 mL per minute. Collect 30 fractions, 5 mL each.

[0123] Assay the HPLC fractions in the *V. harveyi* BB170 AI-2 assay, and pool the active fractions.

[0124] The product from the C<sub>18</sub> column contains the signaling molecule and a small number of other organic molecules. This purified preparation of the signaling molecule has activity 50-100 times greater than that of the partially purified material described above (the preparation of which did not include the high osmoticum step or the final HPLC step), *i.e.*, 1-10 µg material stimulates a 1,000-fold increase in luminescence in the *V. harveyi* bioassay.

[0125] Subsequent strategies for purifying the AI-2 analog have led to the identification of a novel *in vitro* system for producing AI-2. Thus, in addition to providing a cloned, overexpressed and purified *S. typhimurium* LuxS protein, the present invention also provides a method for producing AI-2 *in vitro*. The present invention provides a mechanism for generating large quantities of pure AI-2 useful for mass spectral and NMR analysis, and for screening compounds that regulate the activity of AI-2. Moreover, the present invention provides a method for determining the *in vivo* biosynthetic pathway for AI-2 synthesis. The *in vitro* method for AI-2 analog production is described below in Example 5 and Figure 15. The method provides a novel means for efficiently producing autoinducer molecules for further study. The method also provides a means for producing substantial quantities of AI-2 analogs for use in commercial applications. Such applications include, but are not limited to, adding AI-2 analogs to a growth medium to increase bacterial growth. Such a method is particularly useful in the production of antibiotics from cultured bacteria. Preferably, the AI-2 analog is produced by the *in vitro* method set forth in Example 5 of the disclosure.

#### Uses of AI-2 Analogs

[0126] The autoinducer-2 analogs described here are useful for influencing quorum sensing in bacteria. Such analogs may be identified by large-scale screening of a variety of test compounds through use of the *V. harveyi* bioassay. Reduction in signaling activity in the presence of a test compound indicates the ability of that compound to, for example, block bacterial pathogenesis by affecting the expression of one or more virulence factors.

[0127] For example, naturally produced compounds can be screened for their effect on the interaction between autoinducer-2 and receptors for it, such as LuxP and LuxP-LuxQ.

[0128] Now that components of the quorum sensing pathway have been identified in *E. coli*, inhibition of one or more of those components can be used to screen potential signaling molecule inhibitors or analogs. The inventors have prepared a *ler-lacZ* reporter fusion construct to be used in testing for reduction of expression of the Type III secretion

gene in *E. coli* O157:H7 (pathogenic strain) directly. Furthermore, a similar locus exists in *S. typhimurium*.

[0129] Thus, the invention provides a method for selecting autoinducer-2 analogs such as 4-hydroxy-5-methyl-2h-furan-3-one by measuring the activity of a selected gene by the naturally-occurring autoinducer-2 in the presence and absence of a suspected inhibitor or synergist. In this manner, compounds that regulate bacterial pathogenesis can be rapidly screened.

[0130] Compounds identified in the method of the invention can be further evaluated, detected, cloned, sequenced, and the like, either in solution or after binding to a solid support, by any method usually applied to the detection of a specific DNA sequence such as PCR, oligomer restriction (Saiki et al., Bio/Technology, 3:1008-1012, 1985), allele-specific oligonucleotide (ASO) probe analysis (Conner et al., Proc. Natl. Acad. Sci. USA, 80:278, 1983), oligonucleotide ligation assays (OLAs) (Landegren et al., Science, 241:1077, 1988), and the like. Molecular techniques for DNA analysis have been reviewed (Landegren et al., Science, 242:229-237, 1988). Also included in the screening method of the invention are combinatorial chemistry methods for identifying chemical compounds that bind to LuxP or LuxQ. See, for example, Plunkett and Ellman, "Combinatorial Chemistry and New Drugs," Scientific American, April, p.69 (1997).

[0131] Naturally-occurring autoinducer-2 can be purified from the native source using conventional purification techniques, derived synthetically by chemical means, or preferably, produced by the *in vitro* method of the invention described below.

[0132] One of the instant methods entails regulating the activity of an autoinducer-2 receptor comprising contacting an autoinducer-2 receptor with an AI-2 agonist or antagonist. A further embodiment of this method employs a compound of Formulas I, II, III, or IV of any combination thereof. A further embodiment of either of these embodiments entails when the autoinduced-2 receptor is selected from the group consisting of Lux P and Lux Q. Further embodiments of any of the foregoing embodiments occur when the AI-2 receptor is found on a bacterial cell, and in turn when the bacterial cell is found in a warm-blooded host.



[0133] The activity regulated in any of the foregoing embodiments can be, for example, bacterial cell growth, bacterial virulence, siderophore expression, exopolysaccharide production in bacterial cells, bacteria colony morphology (including smooth colony morphology, such as that exhibited by a pathogenic bacterial cell), biofilm formation, and the like. These embodiments can be practiced with compounds from Formulas I, II, III, and IV, or any combination thereof, and especially so with 4-hydroxy-5-methyl-3(2H)furanone. One embodiment of any of the foregoing embodiments can be practiced wherein AI-2 receptor is part of a bacterial cell selected from the group consisting of *Vibrio harveyi*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Pseudomonas phosphoreum*, *Yersinia enterocolitica*, *Escherichia coli*, *Salmonella typhimurium*, *Haemophilus influenzae*, *Helicobacter pylori*, *Bacillus subtilis*, *Borrelia burgdorferi*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Yersinia pestis*, *Campylobacter jejuni*, *Deinococcus radiodurans*, *Mycobacterium tuberculosis*, *Enterococcus faecalis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes* and *Staphylococcus aureus*.

[0134] The invention further provides methods of inhibiting the infectivity of a pathogenic organism as well as therapeutic compositions containing an AI-2 analog or AI-2 inhibitor of the invention. The methods comprise administering to a subject a therapeutically effective amount of a pharmaceutical composition that inhibits the activity of naturally-occurring AI-2.

[0135] A pharmaceutical composition of the invention can include a compound as set forth in Structure I, II, III or IV. A composition including such a compound can, for example, prevent the transcriptional activation of extracellular virulence factors such as accessory cholera enterotoxin, adenylate cyclase toxin, adhesin, aerolysin toxin, aggregation substance, i.e., asa373, Agr A,B,C,D, SigB etc, alkaline protease, alpha toxin, alpha-haemolysin, alveolysin, anthrax toxin, APX toxin, beta toxin, botulinum toxin, bundle forming pilus structural subunit, C2 toxin, C3 toxin, C5A peptidase, cardiotoxin, chemotaxis, cholera toxin, ciliotoxin, clostridial cytotoxin, clostridial neutotoxin, collagen adhesion gene, crystal endotoxin, cyaA toxin, cytolysin, delta toxin, delta toxin, delta-lysin, diphtheria toxin, emetic toxin, endotoxin, staphylococcal enterotoxins A, B, C1, C2, C3, D, E, G, enterotoxin, exfoliative toxin, exotoxin, exotoxin A, exotoxin B, exotoxin C, extracellular elastase,

fibrinogen, fibronectin binding protein, i.e., fnbA, filamentous hemagglutinin, fimbriae, gamma hemolysin, gelatinase, i.e., gelE, haemolysin, hemolysin B, hemagglutinin, histolyticolysin, IGG binding protein A, i.e., spaI, intimin, invasin, iron siderophores, ivanolysin, ivanolysin O, lantibiotic modifying enzyme, lantibiotic structural protein, lecithinase, ler (positive regulator of LEE genes), leukotoxin, lipoprotein signal peptidase, listeriolysin O, M protein, motility, neurotoxin, nonfimbrial adhesins, oedema factor, perfringolysin O, permease, pertussis toxin, phospholipase, pili, plasmid encoded regulator per, pneumolysin, poly-D-glutamic acid capsule, pore-forming toxin, proline permease, RNAlII, RTX toxin, serine protease, shiga toxin, siderophore/iron acquisition protein, SigA proteases, Spe A, Spe B, Spe C, Sta toxin, Stb toxin, streptolysin O, streptolysin S, superantigen, superoxide dismutase, TCP, tetanus toxin, thiol-activated cytolysin, tracheal cytotoxin, TSST toxin (TSST-1), urease and zona occludens toxin.

[0136] The invention provides autoinducer-2 analogs that influence bacterial cell growth or the expression of a bacterial virulence factor by regulating the activity of pathways activated by naturally-occurring autoinducer-2. Such pathways include, for example, the production of siderophores and the regulation of bacterial colony morphology. An autoinducer-2 analog of the present invention can be used to increase bacterial growth by acting in concert with, or as a replacement for, AI-2. Alternatively, an autoinducer-2 analog can inhibit AI-2 by competing with naturally-occurring AI-2 for binding to autoinducer-2 receptors including LuxP or LuxQ.

[0137] In addition, autoinducer-2 receptors such as LuxP or LuxQ provide a common target for the development of a vaccine. Antibodies raised to autoinducer-2 receptors, such as LuxP or LuxQ, for example, can inhibit the activation of bacterial pathways associated with virulence. Autoinducer-2 receptors provide common antigenic determinants that can be used to immunize a subject against multiple pathogen-associated disease states. Challenging a subject with a polypeptide derived from an autoinducer-2 receptor, or an antigenic fragment thereof, isolated from a particular organism may confer protective immunity to other disease states associated with a different organism. For example, a vaccine developed to the LuxP protein isolated from *V. cholerae* may be capable

of cross-reacting with a LuxP homologue expressed by a different organism. Thus methods of the present invention can be used to treat pathogen-associated disease states.

[0138] The invention further provides a method for promoting production of a bacterial product, such as an antibiotic or virulence factor, by adding an autoinducer-2 analog to a culture of bacteria at a concentration effective to promote cellular metabolism, growth or recovery. For example, antibiotic-producing bacteria typically only produce an antibiotic near the peak of log phase growth. Addition of autoinducer-2 analogs to a culture of such bacteria provides a method for inducing antibiotic production at an earlier phase of growth, and/or for increasing the amount of antibiotic produced.

[0139] The invention further provides a method for identifying factors that degrade or inhibit synthesis autoinducer-2. The concentration of naturally-occurring autoinducer-2 reaches a maximum in early log phase of bacterial cell growth and subsequently decreases in late log phase and stationary phase. These data indicate that a mechanism exists for the degradation of autoinducer-2 at a specific point in bacterial growth. The invention enables identification of the mechanism that controls levels of naturally-occurring autoinducer-2. For example, partially purified bacterial extracts can be assayed against isolated autoinducer-2 to identify those fractions that degrade autoinducer-2.

[0140] Autoinducer-2 analogs can also be used in screens for other targets that they regulate. Cloned promoter-fusion libraries can be prepared from any species of bacteria and these libraries can be used to identify genes that are affected by the signaling factor, simply by screening for differences in reporter activity in the presence and absence of an autoinducer-2 analog.

#### Description of Nucleic Acids Encoding Proteins Involved in Signaling Factor Biosynthesis

[0141] The applicants have cloned and characterized the genes that encode a novel family of proteins responsible for autoinducer production in *V. harveyi*, *S. typhimurium* and *E. coli*. Members of this family of autoinducer production genes are designated as *luxS*, specifically *luxS<sub>V.h.</sub>*, *luxS<sub>S.t.</sub>*, and *luxS<sub>E.c.</sub>*, for *E. coli*, *V. harveyi*, *S. typhimurium* and *E. coli.*, respectively, and their sequences appear as SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NOS:3 and 4, respectively (the sequences read in the 5' to 3' direction). Corresponding amino acid

sequences appear as SEQ ID NO:10, SEQ ID NO:11 and SEQ ID NO:12, respectively (and in Figure 12). It is believed that SEQ ID NOS:1 and 2 constitute full-length clones, whereas SEQ ID NO:3 and SEQ ID NO:4 do not.

[0142] This invention encompasses *luxS* genes and their encoded enzymes from any bacterial species, having the sequence, structural and functional properties of the *luxS*-encoded proteins described herein. As mentioned in Example 3, homologous nucleic acid sequences have been identified in a variety of bacterial species, but their identity as *luxS* genes heretofore had not been appreciated. *luxS* nucleotide and deduced amino acid sequences from other bacterial species appear as SEQ ID NOS: 5-9 and 13-17, respectively, and include sequences from *Haemophilus influenzae*, *Helicobacter pylori*, *Bacillus subtilis*, *Borrelia burgdorferi* and *Vibrio cholerae*.

[0143] Variants and natural mutants of SEQ ID NOS:1-9 are likely to exist within different species or strains of *Vibrio*, *Escherichia* and *Salmonella* (indeed, *E. coli* strain DH5 $\alpha$  possesses a non-functional mutant form of the gene). Because such variants are expected to differ in nucleotide and amino acid sequence, this invention provides an isolated *luxS* nucleic acid and encoded protein having at least about 50-60% (preferably 60-80%, most preferably over 80%) sequence homology in the coding region with the nucleotide sequences set forth as SEQ ID NOS:1-9, respectively (and, preferably, specifically comprising the coding regions of SEQ ID NOS:1-9), and the amino acid sequence of SEQ ID NOS:10-17. Because of the natural sequence variation likely to exist among these proteins and nucleic acids encoding them, one skilled in the art would expect to find up to about 40-50% sequence variation, while still maintaining the unique properties of the LuxS-encoded proteins of the present invention. Such an expectation is due in part to the degeneracy of the genetic code, as well as to the known evolutionary success of conservative amino acid sequence variations, which do not appreciably alter the nature of the protein. Accordingly, such variants are considered substantially the same as one another and are included within the scope of the present invention.

[0144] The *luxS* genes identified here bear no homology to other genes known to be involved in production of acyl-homoserine lactone autoinducers (*luxI*-like (Fuqua *et al.*, J. Bacteriol. 176, 269-275, 1994), *luxLM-ainS*-like (Bassler *et al.*, 1993, *supra*; Gilson *et al.*, J.

Bacteriol. 177, 6946-6951, 1995). Database analysis of bacterial genomes reveals that many other species of bacteria possess a gene homologous to *luxS* from *V. harveyi*, *S. typhimurium* and *E. coli*, as shown in Table 1.

Table 1

Bacterium	Percent homology to LuxS of <i>V. harveyi</i>	Percent identity to LuxS of <i>V. harveyi</i>
<i>Haemophilus influenza</i>	88	72
<i>Helicobacter pylori</i>	62	40
<i>Bacillus subtilis</i>	58	38
<i>Borrelia burgdorferi</i>	52	32
<i>Neisseria meningitidis</i>	89	80
<i>Neisseria gonorrhoeae</i>	89	80
<i>Yersinia pestis</i>	85	77
<i>Campylobacter jejuni</i>	85	74
<i>Vibrio cholerae</i>	95	90
<i>Deinococcus radiodurans</i>	65	45
<i>Mycobacterium tuberculosis</i>	59	41
<i>Enterococcus faecalis</i>	60	44
<i>Streptococcus pneumoniae</i>	57	36
<i>Streptococcus pyogenes</i>	57	36.

[0145] Mutagenesis of *luxS* in *V. harveyi*, *S. typhimurium* and *E. coli* eliminates production of the signaling molecule in all three species of bacteria. *S. typhimurium* could be complemented to full production of the molecule by the introduction of either the *E. coli* O157:H7 *luxS*<sub>E.c.</sub> gene or the *V. harveyi* BB120 *luxS*<sub>V.h.</sub> gene. These results indicate that both the *E. coli* and *V. harveyi* LuxS proteins can function with *S. typhimurium* cellular components to produce the signaling molecule. Introduction of either the *E. coli* O157:H7

luxS<sub>E.c.</sub> or the *V. harveyi* BB120 luxS<sub>V.h.</sub> gene partially complemented *E. coli* DH5 $\alpha$  to produce naturally-occurring autoinducer-2.

[0146] Pathogenicity is connected with quorum sensing. Pathogenic and non-pathogenic strains differ in their production of naturally-occurring autoinducer-2. For example, *E. coli* O157:H7 strains produce naturally-occurring autoinducer-2 with or without glucose while *E. coli* K-12 strains do not produce it in the absence of a preferred carbon source. Furthermore, all of the *E. coli* O157 strains tested produce greater signaling activity than do non-pathogenic *E. coli* strains. Similarly, pathogenic *S. typhimurium* 14028 produces significantly more signaling activity than does *S. typhimurium* LT2.

#### Preparation of LuxS Nucleic Acids, Encoded Proteins, and Immunologically Specific Antibodies

[0147] **Nucleic Acids.** Nucleotide sequence information, such as the DNAs having SEQ ID NOS:1-9, enables preparation of an isolated nucleic acid molecule of the invention by oligonucleotide synthesis. Synthetic oligonucleotides may be prepared by the phosphoramidite method employed in the Applied Biosystems 38A DNA Synthesizer or similar devices. The resultant construct may be purified according to methods known in the art, such as high performance liquid chromatography (HPLC). Long, double-stranded polynucleotides, such as a DNA molecule of the present invention, must be synthesized in stages, due to the size limitations inherent in current oligonucleotide synthetic methods. Such long double-stranded molecules may be synthesized as several smaller segments of appropriate complementarity. Complementary segments thus produced may be annealed such that each segment possesses appropriate cohesive termini for attachment of an adjacent segment. Adjacent segments may be ligated by annealing cohesive termini in the presence of DNA ligase to construct an entire 1.8 kb double-stranded molecule. A synthetic DNA molecule so constructed may then be cloned and amplified in an appropriate vector.

[0148] *LuxS* nucleic acids also may be isolated from appropriate biological sources through use of known methods. In a preferred embodiment, a genomic clone is isolated from a cosmid expression library of an *S. typhimurium* or *E. coli* genome, or a genomic clone is isolated from a cosmid library of another bacterial genome.

[0149] Nucleic acids having the appropriate level sequence homology with the protein coding region of any of SEQ ID NOS:1-9 may be identified by use of hybridization and washing conditions of appropriate stringency. For example, hybridizations may be performed, according to the method of Sambrook et al., through use of hybridization solution comprising: 5X SSC, 5X Denhardt's reagent, 1.0% SDS, 100 g/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. After hybridization at 37-42°C for at least six hours, filters are washed as follows: (1) five minutes at room temperature in 2X SSC and 1% SDS; (2) fifteen minutes at room temperature in 2X SSC and 0.1% SDS; (3) thirty minutes to one hour at 37°C in 1X SSC and 1% SDS; (4) 2 hours at 42-65° in 1X SSC and 1% SDS, changing the solution every 30 minutes.

[0150] *LuxS* nucleic acids can also be found through a search of publicly available databases for the *luxS* sequence in the bacterial genome of interest, design of PCR primers from the sequence, amplification of the gene directly from the chromosome, and cloning of the PCR product. Alternatively, if the complete sequence of a specific bacterial genome is not available, the sequences set forth in the present invention, or any other *luxS* sequence, may be used to design degenerate oligonucleotides for PCR amplification and cloning of *luxS* from the chromosome.

[0151] Nucleic acids of the present invention may be maintained as DNA in any convenient cloning vector. In a preferred embodiment, clones are maintained in plasmid cloning/expression vector, such as pBluescript (Stratagene, La Jolla, CA), which is propagated in a suitable *E. coli* host cell.

[0152] *LuxS* nucleic acids of the invention include DNA, RNA, and fragments thereof, which may be single or double-stranded. Thus, this invention provides oligonucleotides (sense or antisense strands of DNA or RNA) having sequences capable of hybridizing with at least one sequence of a nucleic acid molecule of the present invention, such as selected segments of the DNA having SEQ ID NOS:1, 2 or 3. Such oligonucleotides are useful as probes for detecting *luxS* genes or transcripts.

[0153] **Proteins and Antibodies.** A full-length *luxS* gene product of the present invention may be prepared in a variety of ways, according to known methods. The protein

may be purified from appropriate sources, e.g., cultured bacteria such as *S. typhimurium*, *E. coli* or *V. harveyi*.

[0154] The availability of full-length *luxS* nucleic acids enables production of the encoded protein through use of *in vitro* expression methods known in the art. According to a preferred embodiment, the enzyme may be produced by expression in a suitable expression system. For example, part or all of a DNA molecule, such as the DNA having SEQ ID NO:1 or 2, may be inserted into a plasmid vector adapted for expression in a bacterial cell, such as *E. coli*, or a eucaryotic cell, such as *Saccharomyces cerevisiae* or other yeast. Such vectors comprise the regulatory elements necessary for expression of the DNA in the host cell, positioned in such a manner as to permit expression of the DNA in the host cell. Such regulatory elements required for expression include promoter sequences, transcription initiation sequences and, optionally, enhancer sequences.

[0155] The protein produced by *luxS* gene expression in a recombinant procaryotic or eucaryotic system may be purified according to methods known in the art. In a preferred embodiment, a commercially available expression/secretion system can be used, whereby the recombinant protein is expressed and thereafter secreted from the host cell, to be easily purified from the surrounding medium. If expression/secretion vectors are not used, an alternative approach involves purifying the recombinant protein by affinity separation, such as by immunological interaction with antibodies that bind specifically to the recombinant protein. Such methods are commonly used by skilled practitioners.

[0156] The protein encoded by the *luxS* gene of the invention, prepared by one of the aforementioned methods, may be analyzed according to standard procedures. For example, the protein may be subjected to amino acid sequence analysis, according to known methods. The stability and biological activity of the enzyme may be determined according to standard methods, such as by the ability of the protein to catalyze production of the signaling molecule under different conditions.

[0157] The present invention also provides antibodies capable of immunospecifically binding to the *luxS*-encoded protein of the invention.

#### Uses of LuxS Nucleic Acids, Encoded Protein and Immunologically Specific Antibodies



[0158] *LuxS* nucleic acids or fragments thereof may be used as probes for *luxS* genes in (1) *in situ* hybridization; (2) Southern hybridization (3) northern hybridization; and (4) assorted amplification reactions such as polymerase chain reactions (PCR). They may also be used as probes to identify related genes from other bacteria.

[0159] *LuxS* nucleic acids may further be used to produce large quantities of substantially pure encoded protein, or selected portions thereof. Furthermore, the cloned genes inserted into expression vectors can be used to make large quantities of the signaling molecule itself, from any selected bacterial species, in a recombinant host such as *E. coli* DH5 $\alpha$ . Cloning of specific *luxS* genes, and production of a large quantity of the encoded protein, thereby allows production of a large quantity of the specific autoinducer. This capability will be particularly useful in determining differences, if any, in the structures of autoinducers from different species. Alternatively, large quantities of signaling molecule from the species of interest could be made through use of the cloned gene in an expression vector, and thereafter used in library screens for potential targets in Petri plate assays.

[0160] Purified *luxS* gene products, or fragments thereof, may be used to produce antibodies that also may serve as sensitive detection reagents for those proteins in cultured cells. Recombinant techniques enable expression of fusion proteins containing part or all of a selected *luxS*-encoded protein. The full length protein or fragments of the protein may be used to advantage to generate an array of antibodies specific for various epitopes of the protein, thereby providing even greater sensitivity for detection of the protein. Other uses of the LuxS proteins include overproduction to make a quantity sufficient for crystallization. Knowledge of the crystal structure of the LuxS proteins would enable determination of the LuxS active site that produces the naturally-occurring autoinducer-2, and that could therefore be used for computer-aided design of autoinducer-2 analogs, LuxS inhibitors, and rational drug design.

[0161] Antibodies specific for a LuxS-encoded protein may be used in a variety of assays designed to detect and quantitate the protein. Such assays include, but are not limited to: (1) flow cytometric analysis; (2) immunochemical localization of a LuxS protein in cells or tissues; and (3) immunoblot analysis (e.g., dot blot, Western blot) of extracts from various

cells and tissues. Additionally, as described above, antibodies can be used for purification of the proteins (e.g., affinity column purification, immunoprecipitation).

#### Vibrio Harveyi Screening Strain

[0162] The invention provides a *luxN*- and *luxS*- strain of *V. harveyi*, designated MM32, for identifying inhibitors of the quorum-sensing pathway. Since the new strain is *luxN*- (i.e., sensor 1-), AI-1 does not affect its ability to grow or to luminesce. Further, since MM32 cannot produce AI-2, addition of exogenous AI-2 or its analogs allows rapid identification of inhibitors of AI-2.

[0163] *V. harveyi* strains containing separate mutations in Lux genes L or M, or in Lux genes S or Q, lack the ability to synthesize or detect autoinducer-1 or autoinducer-2, respectively. Use of strain BB170, which is sensor 1-, sensor 2+ (LuxN-, LuxQ+) allows detection of autoinducer-2 in diverse bacteria. The light emission response of wild type, LuxN- and LuxQ- phenotypes to increasing cell density is shown in Figure 14.

[0164] The strains described above are ideally suited for the preparation of a kit. Such a kit may comprise a carrier compartmentalized to receive one or more containers such as vials, tubes, and the like, each of the containers comprising one of the separate elements to be used in the method. The container may comprise a strain of bacteria capable of detecting the presence of an autoinducer. Preferably, the bacterial strain is capable of providing a signal in the presence of autoinducer-2. More preferably, the desired strain lacks the ability to detect AI-1 (sensor 1-) and to synthesize AI-2. Thus, the kit may provide a *luxN*- and *luxS*- strain of *V. harveyi* designated MM32 for identifying autoinducer-2 as well as inhibitors of autoinducer-2 and the quorum-sensing pathway.

#### Methods for Detecting a Bacterial Biomarker

[0165] The present invention provides methods of using autoinducer-2 analogs to identify and regulate the expression of bacterial biomarkers in bacteria. The method is particularly useful for identifying a biomarker of pathogenic bacteria, which are thought to express an antigenic determinant or other biomarker in response to increased autoinducer concentration in the surrounding environment. Thus the present invention provides a method

for identifying such a biomarker by exposing a bacterium to an autoinducer-2 analog and assaying for the presence of the biomarker through use of a labeled nucleic acid or other biomolecule, such as an antibody.

[0166] The method can be used in a screen to detect infections, for example. In addition, the method of the invention can be used to analyze differential gene expression in a bacterial cell following contact with an autoinducer through comparison of the expression of genes in different cells.

[0167] The materials described above are ideally suited for the preparation of a kit. Such a kit may comprise a carrier compartmentalized to receive one or more containers such as vials, tubes, and the like, each container comprising one of the separate elements to be used in the method. A kit of the invention may contain a first container comprising isolated autoinducer-2. The isolated autoinducer-2 can be used to regulate the expression of a biomarker in a target bacterium. For example, autoinducer-2 can be used to induce expression of a particular biomarker that can then be identified by a probe. Thus, the kit may contain a second container comprising a probe that can be detectably labeled. The kit may also have a third container comprising a reporter, such as a biotin-binding protein, such as avidin or streptavidin, bound to a label, such as an enzymatic, fluorescent, or radionuclide label. Other reporters and labels are well known in the art. For example, the kit of the invention may provide reagents necessary to perform nucleic acid hybridization analysis as described herein or reagents necessary to detect antibody binding to a target.

#### Regulation of Biofilm Formation

[0168] Bacteria can adhere to solid surfaces to form slimy, slippery coatings known as biofilms, which are a highly hydrated matrix of exopolymers, typically polysaccharide, and other biopolymers. The biofilm mode of existence, involving colony-forming sessile bacteria, accounts for a sizable portion of the biomass in many environments. In some instances, biofilm-associated forms of bacteria may outnumber their planktonic (free-swimming) equivalents by several orders of magnitude. The transition from a planktonic existence to growth attached to a surface occurs in response to many environmental factors, including long-term growth under conditions of nutrient deprivation

or high osmolarity. Biofilms may contain either a single species, or multiple species of bacteria, and these can be organized into higher order structures (e.g. water/nutrient channels, cellular pillars, dense monolayers punctuated by microcolonies) that benefit the entire community. Biofilm colonies exhibit coordinated metabolic responses, such as spatially distinct gene expression in different regions of the biofilm, that contribute to their overall fitness. Biofilms allow bacteria to survive in hostile environments.

[0169] Bacterial biofilms are at the root of many persistent and chronic bacterial infections. Consequently, the prevention of colonization, and eradication of biofilm-associated bacteria is a major goal of modern medicine. Unlike single cells, biofilm formation can withstand exposure to most biocidal treatments. In the biofilm, the extracellular matrix (glycocalyx) provides a barrier that protects and isolates the microorganisms from biocides such as surfactants and antibiotics. In one study, biofilm cultures were able to withstand a dose of antibiotic twenty-fold greater than needed to eradicate planktonic cultures of the same bacteria. It also shields the resident microorganisms from host defense mechanisms such as antibodies and antigen processing by phagocytes.

[0170] Biofilm-associated infections have been implicated in a number of disease conditions. Examples include prostaticitis, osteomyelitis, septic arthritis, and cystic fibrosis. In some of these diseases (e.g. septic arthritis), the biofilm can cause destruction of the host surface structure. In many of these conditions, the biofilm provides a reservoir of bacteria that can lead to chronic and recurrent infections (sepsis). Biofilm formation can also be a complication in bioimplants, such as bone prostheses, heart valves, and pacemakers. Biofilm formation may compromise the function of the implant, or lead to serious bone or joint infections.

[0171] Because quorum sensing strongly influences biofilm formation, quorum-sensing blockers can impede formation of biofilms and substantially decrease the extent of biofilms that have already formed on a surface. Thus, by providing the structure of autoinducer-2 (AI-2) and analogs of AI-2, such as Structure I, II, III or IV, the present invention provides a new approach to inhibiting bacterial infections by regulating biofilm formation.

[0172] The invention further provides data indicating that AI-2 regulates bacterial biofilm formation. Null mutant strains of *V. harveyi* lacking autoinducer-1 and autoinducer-2 activity (AI-1-/AI-2-) do not produce biofilm, unlike wild type, AI-1-/AI-2+ and AI-1+/AI-2- *V. harveyi* strains. Addition of AI-1, AI-2 or *Salmonella* AI-2 to the medium allows (AI-1-/AI-2-) *V. harveyi* to produce biofilm. Thus, the invention provides a way of regulating biofilm formation by contacting a bacterial cell with autoinducer-2 or an autoinducer-2 analog.

[0173] Furthermore, the unexpected finding that quorum-sensing blockers inhibit biofilm formation implies that compounds that exhibit quorum-sensing blocking in other systems, such as protease production, will also inhibit biofilm formation.

[0174] In another embodiment, the invention provides a method of removing a biofilm from a surface that comprises treating the surface with a compound of the invention. The surface is preferably the inside of an aqueous liquid distribution system, such as a drinking water distribution system or a supply line connected to a dental air-water system, where removal of biofilms can be particularly difficult to achieve. The compound is preferably applied to the surface either alone or together with other materials such as conventional detergents or surfactants.

[0175] A further embodiment of the invention is an antibacterial composition comprising a compound of the invention together with a bacteriocidal agent. In the antibacterial compositions, the compound of the invention helps to remove the biofilm while the bacteriocidal agent kills the bacteria. The antibacterial composition is preferably in the form of a solution or suspension for spraying and/or wiping on a surface.

[0176] In yet another aspect, the invention provides an article coated and/or impregnated with a compound of the invention in order to inhibit and/or prevent biofilm formation thereon. The article is preferably composed of plastic with the compound of the invention distributed throughout the material.

### Synergistic Antibiotic Compositions

[0177] In a further embodiment, the invention provides a synergistic antibiotic composition comprising an antibiotic and an inhibitor of the quorum-sensing pathway of a microorganism, or a pharmaceutically-acceptable salt thereof, and one or more pharmaceutically acceptable carriers, adjuvants or vehicles.

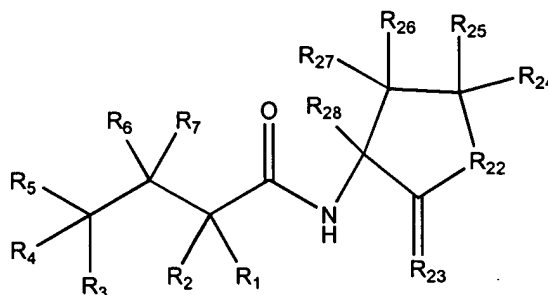
[0178] The synergistic antibiotic composition of the invention can be used to treat infections in a warm-blooded animal caused by microorganisms possessing a quorum-sensing mechanism, which comprises administering to the animal a therapeutically effective amount of the synergistic antibiotic composition of this invention.

[0179] Surprisingly, we have found that combinations of inhibitors for one or more of the quorum sensing pathways with antibiotics effective against microorganisms containing the quorum sensing mechanism reduces the amount of antibiotic normally necessary for its therapeutic effect. Also, the combination may lower the therapeutically effective amount of the antibiotic so that it has a beneficial effect against the microorganism when ordinarily it was considered to have no effect at concentrations considered both effective and safe. Thus, another aspect of the instant invention is directed to a synergistic antibiotic composition comprising an antibiotic and an inhibitor of the quorum-sensing pathway of a microorganism. By “synergistic”, we mean that the combination of the quorum sensing inhibitor lowers the dose of the antibiotic required to kill (in a bactericidal agent) or arrest the growth (in a bacteristatic agent) of the microorganism being treated in warm-blooded animal, or renders the antibiotic effective against the organism in an animal when it ordinarily is not effective.

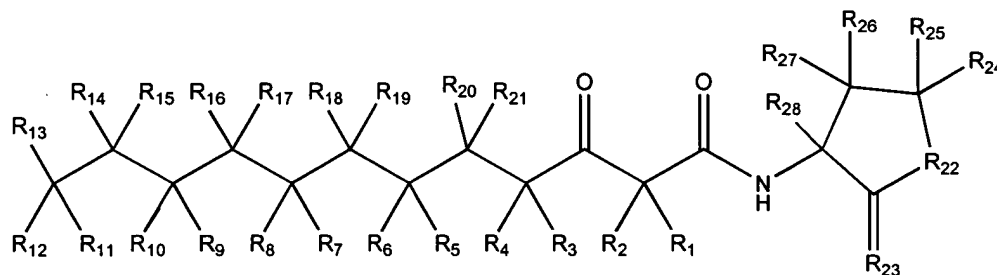
[0180] By “inhibitor of the quorum-sensing pathway of a micro organism” we mean inhibitors that can either inhibit the production, or the response to, an autoinducer. Such inhibitors may inhibit one or more of the AI-1, AI-2 and the peptide-mediated quorum sensing pathways. For instance, inhibitors of the AI-2 pathway (depicted in Figure 13) are molecules that either bind to or inhibit the production of the proteins derived from the LuxP, LuxS and LuxQ genes of the *V. harveyi* and their homologs in other species. The assays for detecting inhibitors of the AI-1, AI-2 and peptide-mediated sensing pathways appear in Examples 6, 4, and 7, respectively. For the AI-2 pathway, inhibitors are found by their

inhibition of bioluminescence of mutant strains *V. harveyi* that are otherwise insensitive to the effects of AI-1. Inhibitors of the AI-1 pathway are also measured by their ability to inhibit bioluminescence of *V. fischeri*. Finally, inhibitors of the peptide-mediated quorum sensing pathway are detected by their ability diminish the production of the virulence factor  $\delta$ -toxin from *Staphylococcus aureus* as colonies of this bacteria grew to confluence.

[0181] Examples of inhibitors of the AI-1 quorum sensing pathway are analogs of N-acyl-L-homoserine lactones in which the N-acyl side chain has been modified, specifically:



[0182] A modified N-buteryl-L-homoserine lactone compound of the structure V:



[0183] or a modified N-(3-oxododecanoyl)-L-homoserine lactone compound of the structure VI:

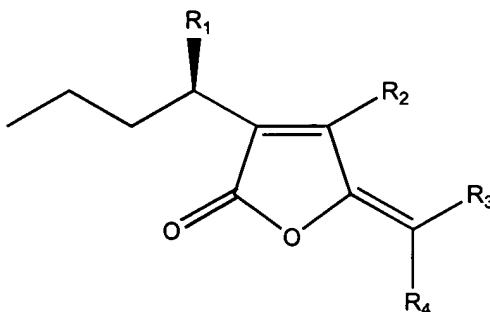
[0184] wherein  $R_1$ -  $R_{22}$  are independently selected from the group consisting of  $C_1$  to  $C_4$  alkyl; a hydrogen atom, hydroxy, amino, and thiol;

[0185]  $R_{22}$  -  $R_{23}$  are independently an oxygen or a sulfur atom; and

[0186]  $R_{24}$  -  $R_{28}$  are independently a hydrogen or a halogen atom. These molecules are described in Davies et al., PCT Patent Application Publication No.

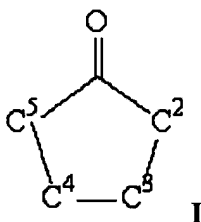
WO98/58075, published December 23, 1998, the entirety of which is incorporated herein by reference.

[0187] Other examples of inhibitors of AI-1 quorum-sensing pathways are halogenated furanones of the structure VII:



[0188] These halogenated furanones are produced by benthic marine macroalga *D. pulchra*. One particular example of such an inhibitor is 4-bromo-5-(bromomethylene)-3-(1-hydroxybutyl)-2-(5H)-furanone. These molecules are described in Manefield et al., Microbiology, 145, 283-291 (1999)).

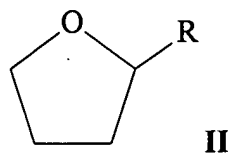
[0189] Examples of the inhibitors of the AI-2 mediated quorum sensing pathway includes compounds of structures I, II, III and IV:



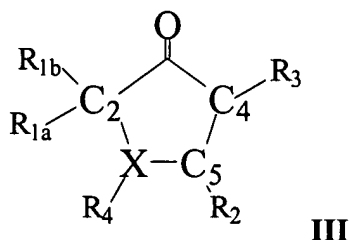
[0190] wherein C<sup>2</sup> is additionally bonded to at least one substituent selected from hydrogen, hydroxyl, C<sub>1-5</sub> alkyl, C<sub>2-5</sub> alkenyl, C<sub>2-5</sub> alkanoyl, C<sub>2-5</sub> alkanoyloxy, heteroaryl, or forms a double bond with an oxygen atom or C<sup>3</sup>; wherein C<sup>3</sup> is additionally bonded to at least one substituent selected from hydrogen, hydroxyl, C<sub>1-5</sub> alkyl, C<sub>2-5</sub> alkenyl, C<sub>2-5</sub> alkanoyl, C<sub>2-5</sub> alkanoyloxy, or forms a double bond with an oxygen atom or C<sup>2</sup>; wherein C<sup>4</sup> is additionally bonded to at least one substituent selected from hydrogen, C<sub>1-5</sub> alkyl, C<sub>2-5</sub> alkanoyl, C<sub>2-5</sub> alkanoyloxy, or forms a double bond with an oxygen atom or C<sup>5</sup>; and wherein C<sup>5</sup> is additionally bonded to at least one substituent selected from hydrogen, C<sub>1-5</sub> alkyl, C<sub>2-5</sub> alkanoyl, C<sub>2-5</sub> alkanoyloxy, or forms a double bond with an oxygen atom or C<sup>4</sup>; wherein at least one of C<sup>2</sup>, C<sup>3</sup>, C<sup>4</sup> or C<sup>5</sup> is bonded to a substituent selected from hydroxyl, C<sub>1-5</sub> alkyl, C<sub>2-5</sub>



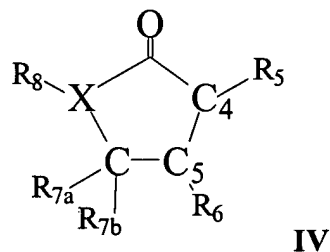
alkenyl, C<sub>2-5</sub> alkanoyl, and heteroaryl; and wherein at most one carbon-carbon double-bond is present in the ring of Structure I;



wherein R is a C<sub>1-5</sub> alkoxy group;

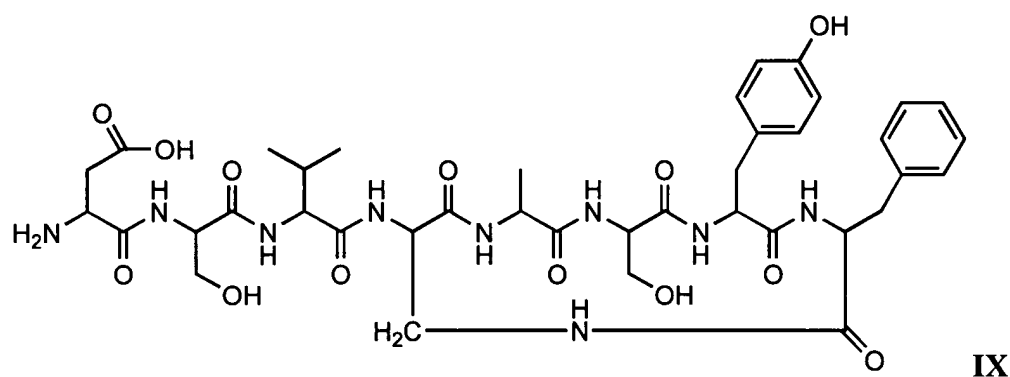
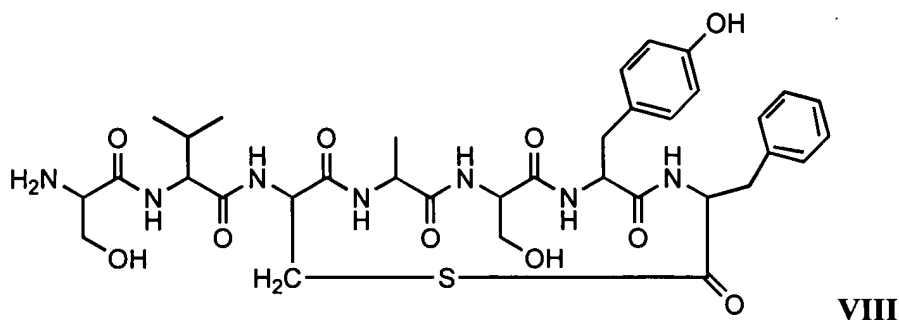


[0191] wherein X is either an oxygen, sulfur or nitrogen atom; R<sub>1a</sub> is either hydrogen, hydroxy, alkyl, acyl, amido, hydroxyl, amino, thio, or aryl; R<sub>1b</sub> is either hydrogen, hydroxy, alkyl, acyl, amido, hydroxyl, amino, mercapto, thio, or aryl, or R<sub>1a</sub> and R<sub>1b</sub> can include the same atom bonded to C<sub>2</sub> to form a double bond with C<sub>2</sub>; R<sub>2</sub> is hydrogen, alkyl, or halogen; R<sub>3</sub> is hydrogen, alkyl, acyl, amido, hydroxyl, amino, thio, or aryl; R<sub>4</sub> is hydrogen or alkyl, if X is nitrogen, or is absent if X is oxygen or sulfur; and wherein C<sub>4</sub> and C<sub>5</sub> can be further joined by a double bond;



[0192] wherein X is oxygen, sulfur or nitrogen; R<sub>7a</sub> is hydrogen, hydroxy, alkyl, acyl, amido, hydroxyl, amino, thio, or aryl; R<sub>7b</sub> is hydrogen, hydroxy, alkyl, acyl, amido, hydroxyl, amino, mercapto, thio, or aryl, or R<sub>7a</sub> and R<sub>7b</sub> can together form a double bond; R<sub>6</sub> is hydrogen, alkyl, or halogen; R<sub>7</sub> is hydrogen, alkyl, acyl, amido, hydroxyl, amino, thio, or aryl; R<sub>8</sub> is hydrogen, if X is nitrogen, or is absent if X is oxygen or sulfur; and wherein C<sub>4</sub> and C<sub>5</sub> can be further joined by a double bond.

[0193] Examples of inhibitors of peptide-mediated quorum sensing molecules include chemically-modified pheromones of *Staphylococcus epidermidis* that are competent inhibitors of the *Staphylococcus aureus* agr (accessory gene regulator) system. These inhibitors include molecules of the structures VIII and IX:



(*cyclo*-SVCASYF)

(*cyclo*-DSV(DAPA)ASYF)

[0194] These modified pheromones were especially effective in inhibiting production of  $\delta$ -toxin in the *S. aureus* strains as described in Otto et al., FEBS Letters, 450 257-262 (1999).

[0195] Further inhibitors of peptide-mediated quorum sensing are compounds of the structure:

(*cyclo*)-YSTCDFIM; (X)

(*cyclo*)-GVNACSSLF; (XI)

(*cyclo*)-GVNASSSLF; (XII) or

(*cyclo*)-GVNA(DAPA)SSLF, (XIII)

[0196] wherein in these four structures the C-terminal carbonyl group forms 1) a thiolactone with the sulfur atom of the cysteine residue (YSTCDFIM and GVNACSSLF); 2)

a lactone group with the oxygen atom of the first serine residue (GVNASSSLF); or 3) an amide bond with amino group of the diaminopropionic acid (DAPA) residue (GVNA(DAPA)SSLF). The synthesis of these molecules and activity of these molecules have been described in Mayville et al., Proc. Natl. Acad. Sci. USA, 96, 1218-1223 (1999).

**[0197]** Further embodiments of the instant synergistic antibiotic composition include those wherein the inhibitor inhibits the AI-1 quorum sensing pathway. Of this latter embodiment, further specific embodiments include those where the inhibitor is a halogenated 2(5H) furanone of Structure VII above, or when the inhibitor is either a modified N-buturyl-L-homoserine lactone of Structure VIII above or a modified N-(3-oxododecanoyl)-L-homoserine lactone of Structure IX above.

**[0198]** Another group of embodiments occurs when the inhibitor of the synergistic antibiotic composition is one that inhibits the AI-2 quorum sensing pathway, and more so when it is of the Structure III or Structure IV above. One further embodiment is when the inhibitor is 2-ethyl-4-hydroxy-5-methyl-3(2H)-furanone. Another group of embodiments for a composition containing an AI-2 inhibitor occurs when 1) the antibiotic is a penicillin, and further when the penicillin is ampicillin; 2) the antibiotic is a quinoline, and further when the quinoline is ciprofloxacin; 3) the antibiotic is vancomycin; and 4) the antibiotic is a sulfonamide, and further when the sulfonamide is sulfisoxazole.

**[0199]** Synergistic antibiotic compositions where the inhibitor inhibits the peptide-mediated quorum sensing pathway constitute another embodiment of the instant synergistic compositions. Within this latter embodiment are those compositions that include inhibitors of the Structure (VIII) and (IX) above, and alternatively those that contain inhibitors of Structures (X) through (XIII) above.

**[0200]** Yet another group of embodiments of the instant synergistic antibiotic compounds occurs when 1) the antibiotic is a penicillin, and further when the penicillin is ampicillin; 2) the antibiotic is a quinoline, and further when the quinoline is ciprofloxacin; 3) the antibiotic is vancomycin; and 4) the antibiotic is a sulfonamide, and further when the sulfonamide is sulfisoxazole.

**[0201]** Further embodiments of the instant pharmaceutical compositions track those set forth above for the synergistic antibiotic compositions of this invention, as determined by the identity of the inhibitors and antibiotics in the pharmaceutical composition.

**[0202]** A further aspect of this invention is directed to a method of treating infections in a warm-blooded animal caused by microorganisms possessing a quorum-sensing mechanism that comprises administering to the animal a therapeutically effective amount of the synergistic antibiotic composition of this invention. It will be understood that the amount of antibiotic administered in the instant method will normally be less than that administered if the inhibitor compound were not present. The amount normally necessary to treat an infection by a particular microorganism can be determined by using the antibiotic susceptibility assay of Example 8, and also in the case of known antibiotics by consulting sources such as J. Hardman and L. Limbard, Editors-in-Chief, Goodman and Gilman's The Pharmacological Basis of Therapeutics, Ninth Edition, McGraw-Hill, New York, (1996), especially at pages 1027 through 1223. Of course, the synergistic antibiotic composition administered in this method can also be in the form of a pharmaceutical composition of this invention. Also, the synergistic antibiotic compound can be administered to warm-blooded animal infected with a microorganism possessing a quorum sensing pathway that would otherwise be refractory to the treatment with the antibiotic alone if screens such as the one set forth in Example 8 below indicate that the synergistic antibiotic composition does indeed have activity against that microorganism.

**[0203]** Further embodiments of this method includes methods for treating infections in warm blooded animals caused by *Streptococcus pyogenes*, and especially so when the inhibitor that is administered is one that inhibits the AI-2 quorum sensing pathway. Of this latter embodiment, further embodiments include those where the antibiotic is vancomycin, when the antibiotic is a quinoline antibiotic, for example, ciprofloxacin; or when the antibiotic is a sulfonamide, for example, sulfisoxazole.

**[0204]** Yet another embodiment of the instant method includes methods for treating infections in warm blooded animals caused by *Staphylococcus aureus*, and especially so when the inhibitor inhibits the AI-2 quorum-sensing pathway. Of this latter embodiment,

further embodiments include those wherein the antibiotic is a penicillin, and in particular when the antibiotic is ampicillin.

#### Medical Devices

[0205] In another embodiment, the compounds and synergistic antibiotic compositions of the invention can be used to inhibit bacterial cell growth and/or biofilm formation on a medical device. Percutaneous devices (such as catheters) and implanted medical devices (e.g., pacemakers, vascular grafts, stents, and heart valves) commonly serve as foci for bacterial infection. The tendency of some microorganisms to adhere to and colonize the surface of the device promotes such infections, which increase the morbidity and mortality associated with use of the devices. Consequently, physicians have great interest in development of surfaces that are less prone to bacterial colonization.

[0206] The synergistic antibiotic compositions of the invention can be used to inhibit bacterial cell growth and biofilm formation on substrates used to manufacture medical devices associated with noninvasive and invasive medical procedures. Such substrates include tubular, sheet, rod and articles of proper shape for use in a number of medical devices such as vascular grafts, aortic grafts, arterial, venous, or vascular tubing, vascular stents, dialysis membranes, tubing or connectors, blood oxygenator tubing or membranes, surgical instruments, ultrafiltration membranes, intra-aortic balloons, stents, blood bags, catheters, sutures, soft or hard tissue prostheses, synthetic prostheses, prosthetic heart valves, tissue adhesives, cardiac pacemaker leads, artificial organs, endotracheal tubes, lenses for the eye such as contact or intraocular lenses, blood handling equipment, apheresis equipment, diagnostic and monitoring catheters and sensors, biosensors, dental devices, drug delivery systems, or bodily implants of any kind. For example, arthroscopic surgery is routinely performed with use of medical devices that minimize the invasiveness of the procedure. Such devices include, for example, ultrathin microfiberoptic endoscopes that offer the laryngologist unique access to the limited spaces of the temporal bone and skull base. In another example, a stent supplemented with a composition of the invention that deters bacterial infections resulting from the presence of the implanted stent can be constructed. Stents are used to maintain an open lumen in tissues including the tracheo-bronchial system,

the biliary hepatic system, the esophageal bowel system, and the urinary tract system. U.S. Patent Number 5,637,113 issued to Tartaglia, and incorporated herein by reference, teaches a stent with a sheet of polymeric film wrapped around the exterior. With regard to the present invention, the film may be loaded or coated with a compound or composition of the invention. Alternatively, the material used to manufacture the stent can be impregnated with a composition of the invention.

[0207] A medical device may be further supplemented with, for example, one or more antibodies, analgesics, anticoagulants, anti-inflammatory compounds, antimicrobial compositions, cytokines, drugs, growth factors, interferons, hormones, lipids, demineralized bone or bone morphogenetic proteins, cartilage inducing factors, oligonucleotides polymers, polysaccharides, polypeptides, protease inhibitors, vasoconstrictors or vasodilators, vitamins, minerals, stabilizers and the like. Supplemented, as used herein, includes medical devices that are impregnated, infused, coated, covered, layered, permeated, attached or connected with a compound or synergistic antibiotic composition of the invention. Methods for immobilizing biomaterials to a medical device are discussed in U.S. Patent Number 5,925,552, which is incorporated herein by reference. Additional methods of coating surfaces of medical devices with antimicrobial compositions are taught in U.S. Pat. No. 4,895,566 (a medical device substrate carrying a negatively charged group having a pKa of less than 6 and a cationic antibiotic bound to the negatively charged group); U.S. Pat. No. 4,917,686 (antibiotics are dissolved in a swelling agent which is absorbed into the matrix of the surface material of the medical device); U.S. Pat. No. 4,107,121 (constructing the medical device with ionogenic hydrogels, which thereafter absorb or ionically bind antibiotics); U.S. Pat. No. 5,013,306 (laminating an antibiotic to a polymeric surface layer of a medical device); and U.S. Pat. No. 4,952,419 (applying a film of silicone oil to the surface of an implant and then contacting the silicone film bearing surface with antibiotic powders). U.S. Pat. No. 5,902,283 further discloses a method for coating a medical device with an antimicrobial agent such that the agent penetrates the exposed surfaces of the device and is impregnated throughout the material of the device.

[0208] The desirability of coating medical devices such as, *inter alia*, surgical implants, sutures and wound dressings with pharmaceutical agents is well documented in the

art. Such coated devices could theoretically provide a means for locally delivering pharmaceutical or therapeutic agents at the site of medical intervention to treat a variety of diseases. For example, surgical implants or sutures coated with antibiotics can provide local delivery of antibiotic directly at an implantation or suture site, thereby decreasing the onset of infection following the surgical intervention. Thus, compounds and synergistic antibiotic compositions of the invention can supplement medical devices used for implantation in guided tissue regeneration (GTR) procedures by preventing or ameliorating infections at the site of tissue regeneration. Such devices and procedures are currently used by those skilled in the medical arts to accelerate tissue regeneration following invasive surgical procedures. For example, nonresorbable or bioabsorbable membranes are used to accelerate tissue regeneration by promoting the repopulation of the wound area with cells that form the architectural and structural matrix of the tissue. The compounds and synergetic antibiotic compositions of the invention can be used to inhibit bacterial growth at the site of tissue regeneration. For example, a compound or synergetic antibiotic composition can be used to aid periodontal tissue regeneration in a human or lower animal by placing a composition containing a bioresorbable polymer, leachable solvent, and a compound or synergetic composition of the invention, at a site in need of periodontal tissue regeneration in a human or other mammal such that the composition is effective for inhibiting bacterial cell growth by releasing a therapeutically-effective amount of the compound or synergetic composition at the site.

[0209] A medical device supplemented with a compound or composition of the invention is further useful for promoting tissue growth during the process of tissue engineering. By “tissue engineering” is meant the creation, design, and fabrication of biological prosthetic devices, in combination with synthetic or natural materials, for the creation, augmentation, or replacement of body tissues and organs. Thus, a medical device supplemented with a compound or composition of the invention includes cell-containing or cell-free device that induce the regeneration of functional human tissues when implanted at a site that requires regeneration. As previously discussed, biomaterial-guided tissue regeneration can be used to promote cell proliferation in, for example, digestive tract tissue for treatment of gastric ulcers or the pathogenic result of Crohn’s disease. A compound or

synergistic antibiotic composition of the invention can be used to promote the growth of reconstituted tissues assembled into three-dimensional configurations at the site of a wound or other tissue in need of such repair by inhibiting pathogenic bacterial cell growth at the site. A compound or synergistic antibiotic composition can be included with the device upon implantation or the device itself can be impregnated with a compound or synergistic antibiotic composition such that the compound or synergistic antibiotic composition is released slowly during tissue regeneration.

[0210] A compound or synergistic antibiotic composition of the invention can be included in external or internal medical devices containing human tissues designed to replace the function of diseased internal tissues. This approach involves isolating cells from the body, placing them on or within structural matrices, and implanting the new system inside the body or using the system outside the body. A compound or synergistic antibiotic composition invention can be included in such matrices to promote the growth of tissues contained in the matrices by inhibiting bacterial growth. For example, compound or synergistic antibiotic composition of the invention can be included in a cell-lined vascular graft to inhibit bacterial cell growth and biofilm formation in the graft. It is envisioned that the invention can be used to augment tissue repair, regeneration, and engineering in products, such as epithelial tissue, cartilage and bone, central nervous system tissues, muscle, liver, and pancreatic islet (insulin-producing) cells.

[0211] A specific, non-limiting example is the use of the invention in promoting the growth of skin graft replacements that are used as a therapy in the treatment of burns and ulcers. Burn victims are particularly susceptible to infections resulting from skin graft treatments. The compounds or synergistic antibiotic composition of the invention can be included during tissue engineering of a skin graft such that the graft is free of bacterial infection when transplanted to a burn victim. Compounds or synergistic antibiotic compositions of the invention can also be included at the site of transplant to further minimize the possibility of infection. Thus, the invention encompasses inhibiting bacterial cell growth and biofilm formation during preparation and application of a tissue graft using an effective amount of a compound or synergistic antibiotic composition of the invention.



[0212] Additionally, or alternatively, the synergistic antibiotic compositions of the invention may be incorporated into a biodegradable carrier. For example, such a carrier is described in U.S. Patent Number 5,788,979. Time-controlled release of the compound or composition is attributable to the degradation or disintegration of the carrier itself, so that the drug or other agent remains captive within the carrier until it is dispensed or released, i.e., freed from its host, by progressive dissolution upon continuing diffusion of the carrier from the reservoir. The compound or composition tends to act locally rather than systemically in such an arrangement.

[0213] It is further envisioned that the synergistic antibiotic compositions of the invention can be used to aid wound repair. For example, U.S. Patent Number 6,117,485 describes a foaming tissue sealant for treating wounded tissue in a subject. The sealant can be formulated to include a compound or composition of the invention. The sealant is useful for significantly diminishing or preventing blood or fluid loss from injured tissues, organs or blood vessels, while also providing a barrier to infection.

[0214] It is further envisioned that the synergistic antibiotic compositions of the invention can be used to inhibit bacterial cell growth and biofilm formation in or on products or devices used for personal hygiene. Soap, toothpaste, dental floss, laundry detergent or moisturizing lotion are examples of consumer products that would benefit from the inclusion of a compound or synergistic antibiotic composition of the invention. In addition, such a compound or composition can be included in a personal hygiene device such as a toothbrush, tongue depressor, or any other such device which comes in contact with a tissue.

[0215] The following description sets forth the general procedures involved in practicing this aspect of the present invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. Unless otherwise specified, general cloning procedures, such as those set forth in Sambrook *et al.*, Molecular Cloning, Cold Spring Harbor Laboratory (1989) (hereinafter “Sambrook *et al.*”) or Ausubel *et al.* (eds) Current Protocols in Molecular Biology, John Wiley & Sons (1998) (hereinafter “Ausubel *et al.*”) are used.

## EXAMPLES

### EXAMPLE 1 - Quorum Sensing in *Escherichia coli* and *Salmonella typhimurium*

[0216] **Assay conditions.** *V. harveyi* reporter strain BB170 (with quorum-sensing phenotype Sensor 1<sup>-</sup>, Sensor 2<sup>+</sup>) can induce *lux* expression only through Signaling System 2. Addition of 10% cell-free spent culture fluid prepared from *V. harveyi* strain BB152 (which contains the autoinducer-2 analog) stimulates a roughly 1000-fold increase in luminescence, an increase that is normalized to 100% activity in Figure 1.

[0217] *E. coli* strain AB1157 and *S. typhimurium* strain LT2 were each grown for 8 h in LB broth or LB broth containing 0.5% glucose and the *E. coli* or *S. typhimurium* cells removed from the growth medium. Addition of 10% cell-free culture fluid maximally induced luminescence in the reporter strain BB170, similar to culture fluids from *V. harveyi* BB152 (Figure 1A). Specifically, *E. coli* AB1157 produced 106% and *S. typhimurium* produced 237% of the *V. harveyi* BB152 activity.

[0218] In control experiments, *E. coli* and *S. typhimurium* grown in LB without added glucose did not produce the signaling factor, nor did blank solutions containing 10% (v/v) of LB medium containing 0.5% glucose. Glucose, amino acids, cAMP, acetate, homoserine lactone, -ketoglutarate and other keto acids that are known to be excreted lacked activity.

[0219] Analogous experiments were performed with the *V. harveyi* reporter strain BB886 (Sensor 1<sup>+</sup>, Sensor 2<sup>-</sup>). *V. harveyi* BB886 is defective in its response to signaling molecules that act through the Signaling System 2 detector, but it is an otherwise wild type strain (Bassler *et al.*, Mol. Microbiol. 13: 273-286, 1994). Figure 1B shows the normalized 100% activation of *V. harveyi* BB886 by cell-free spent culture fluids prepared from *V. harveyi* BB120. *V. harveyi* BB120 produces the System 1 autoinducer *N*-(3-hydroxybutanoyl)-L-homoserine lactone (Bassler *et al.*, 1993, *supra*). Addition of *S. typhimurium* LT2 and *E. coli* AB1157 cell-free culture fluids to *V. harveyi* strain BB886 caused a 5% and a 1% increase above the control level (Figure 1B). Together the results of Figure 1A and 1B show that the signaling molecule produced by *E. coli* and *S. typhimurium* acts specifically through *V. harveyi* Signaling System 2 and not some other, unidentified pathway.

[0220] Figure 2 shows that viable *E. coli* AB1157 and *S. typhimurium* LT2 are Required for Secretion of the Signaling Molecule.

[0221] Growth of *E. coli* AB1157 and *S. typhimurium* LT2 in LB medium containing glucose do not simply remove some pre-existing inhibitor of induction of luminescence. Washed *E. coli* and *S. typhimurium* cells added directly to the luminescence assay.

[0222] *E. coli* AB1157 and *S. typhimurium* LT2 were grown for 8 h in LB containing 0.5% glucose, the cells removed by centrifugation, and the cell pellets washed and resuspended in sterile *V. harveyi* luminescence assay medium. *E. coli* AB1157 or *S. typhimurium* LT2 cells ( $1 \times 10^6$  cells) were added to the diluted *V. harveyi* BB170 culture at the start of the experiment.

[0223] The presence of washed *E. coli* AB1157 or *S. typhimurium* LT2 cells fully induces Lux expression in *V. harveyi* BB170 (Figure 2, left-hand bar in each series)(821-fold and 766-fold, respectively). Identical aliquots of the washed *E. coli* or *S. typhimurium* cells killed with short wave ultraviolet light before addition to the assay did not stimulate luminescence (Figure 2, right-hand bar for each strain). Taken together, the results show that *E. coli* AB1157 and *S. typhimurium* LT2 cells produce the stimulatory factor themselves during the experiment.

[0224] ***E. coli* DH5 $\alpha$  Does Not Produce the Signaling Activity.** Clinical isolates of *E. coli* and *Salmonella* also produce the signaling compound. Ten clinical isolates of *Salmonella* and five pathogenic isolates of *E. coli* O157 were assayed and all produced the activity. It was conceivable that the signal was some normal byproduct of glucose metabolism that simply diffuses out of the cells. This is not the case however, because we show that *E. coli* DH5 $\alpha$ , which is equally capable of utilizing glucose as *E. coli* AB1157 and *S. typhimurium* LT2, does not produce the signaling activity. Figure 1A demonstrates that unlike *E. coli* AB1157 and *S. typhimurium* LT2, the addition of 10% cell-free culture fluid prepared from *E. coli* DH5 $\alpha$  grown 8 h in LB containing 0.5% glucose does not stimulate light production in *V. harveyi* BB170. Similarly, inclusion of washed viable or killed *E. coli* DH5 $\alpha$  cells in the luminescence assay does not stimulate *V. harveyi* BB170 to produce light (Figure 2). The inability of *E. coli* DH5 $\alpha$  to produce the activity indicates that this highly

domesticated strain lacks the gene or genes necessary for either the production or the export of the signaling activity. We assayed other laboratory strains of *E. coli* for the signaling activity (Table 1). Only *E. coli* DH5 $\alpha$  was completely defective in producing the extracellular signal.

[0225] The induction of luminescence in *V. harveyi* reporter strain BB170 by cell-free culture fluids from *V. harveyi*, *S. typhimurium* and *E. coli* is shown. Cell-free culture fluids were prepared from various strains of *V. harveyi*, *S. typhimurium* and *E. coli* as described and tested for production of a signaling substance that could stimulate light production in the reporter strain *V. harveyi* BB170. The level of *V. harveyi* stimulation was normalized to 100%. The data for the 5 h time point are shown.

Table 2

Species and Strain	Induction of Luminescence (%)
<i>V. harveyi</i> BB152	100
<i>S. typhimurium</i> LT2	237
<i>E. coli</i> AB1157	106
<i>E. coli</i> DH5 $\alpha$	5
<i>E. coli</i> JM109	76
<i>E. coli</i> MG1655	100
<i>E. coli</i> MC4100	93

[0226] **Glucose Regulates the Production and Degradation of the Signaling Factor by *S. typhimurium* LT2.** Cell-free culture fluids from *S. typhimurium* LT2 and *E. coli* AB1157 cells grown in LB without added glucose did not stimulate the expression of luminescence in the reporter strain, indicating that metabolism of glucose is necessary for the production of the signal. We tested other carbohydrates, and in general, growth in the presence of PTS sugars (see Postma *et al.*, in *Escherichia coli* and *Salmonella* Cellular and Molecular Biology, (F.C. Niehardt (ed), Am. Soc. Microbiol., Washington D.C., pp. 1149-1174, 1996) enabled *E. coli* AB1157 and *S. typhimurium* LT2 to produce the signal. Of the

sugars tested, growth on glucose induced the synthesis of the highest level of activity. Growth on other carbon sources, for example TCA cycle intermediates and glycerol did not induce significant production of the signaling activity.

[0227] We tested whether the presence of glucose was required for the cells to continue to produce the signal. Figure 3 shows results with *S. typhimurium* LT2 grown in LB containing limiting (0.1%) and non-limiting (0.5%) glucose concentrations. Figure 3A shows that when glucose is limiting, *S. typhimurium* LT2 produces the signal in mid-exponential phase (after 4 h growth), but stops producing the signaling activity once glucose is depleted from the medium. Figure 3B shows that when glucose does not become limiting, *S. typhimurium* LT2 produces greater total activity and continues to produce the signaling activity throughout exponential phase, with maximal activity at 6 h growth. Furthermore, the Figure also shows that the signaling activity synthesized by mid-exponential phase cells is degraded by the time the cells reach stationary phase. In conditions of limiting glucose, no activity remained at stationary phase, and when glucose was plentiful, only 24% of the activity remained. Increasing the concentration of glucose in the growth medium did not change these results, *i.e.*, the activity was secreted during mid-exponential growth, and severely reduced activity remained in the spent culture fluids by stationary phase.

[0228] In sum, the results presented in this example show that *E. coli* and *S. typhimurium* produce a signaling substance that stimulates one specific quorum-sensing system in *V. harveyi*. Many other bacteria have previously been assayed for such an activity, and only rarely were species identified that are positive for production of this factor (Bassler *et al.*, 1997, *supra*). Furthermore, as shown here, the *E. coli* and *S. typhimurium* signal is potent; these bacteria make activity equal to that of *V. harveyi*. The degradation of the *E. coli* and *S. typhimurium* signal prior to stationary phase indicates that quorum sensing in these bacteria is tuned to low cell densities, suggesting that quorum sensing in *E. coli* and *S. typhimurium* is regulated so that the response to the signal does not persist into stationary phase. Additionally, quorum sensing in *E. coli* and *S. typhimurium* is influenced by several environmental factors. The production and the degradation of the signal are sensitive not only to growth phase but also to the metabolic activity of the cells. These results indicate that the quorum-sensing signal in *E. coli* and *S. typhimurium* has two functions; it allows the cells to

communicate to one another their growth phase and also the metabolic potential of the environment.

[0229] Understanding the regulation of quorum sensing in *E. coli* and *S. typhimurium* is important for understanding community structure and cell-cell interactions in pathogenesis. In the wild, pathogenic *E. coli* and *S. typhimurium* may never reach stationary phase because dispersion is critical. It is therefore appropriate that quorum sensing in *E. coli* and *S. typhimurium* should be functioning at low cell density. This situation is in contrast to that of *V. fischeri*, the luminescent marine symbiont, where the quorum-sensing system is only operational at high cell densities; cell densities indicative of existence inside the specialized light organ of the host. The specific quorum-sensing systems of *V. fischeri* and *E. coli* and *S. typhimurium* appear appropriately regulated for the niche in which each organism exists. In both cases, quorum sensing could be useful for communicating that the bacteria reside in the host, not free-living in the environment. Additional complexity exists in the *E. coli* and *S. typhimurium* systems because these bacteria channel both cell density information and metabolic cues into the quorum-sensing circuit. Again, signals relaying information regarding the abundance of glucose or other metabolites could communicate to the bacteria that they should undergo the transition from a free-living mode to the mode of existence inside the host.

[0230] Under all the conditions we have tested, the signaling activity described in this example does not extract quantitatively into organic solvents and it does not bind to either a cation or anion exchange column. Preliminary characterization indicates that the signal is a small (less than 1000 MW) polar but apparently uncharged organic compound. The activity is acid stable and base labile, it is heat resistant to 80 but not 100°C. Purification of the *E. coli*, *S. typhimurium* and *V. harveyi* signal is described in greater detail in the following examples.

#### EXAMPLE 2 - Regulation of Autoinducer Production in *Salmonella typhimurium*

[0231] In this example, the conditions under which *S. typhimurium* LT2 produces AI-2, the extracellular factor that stimulates *lux* expression in the *V. harveyi* Sensor 1<sup>-</sup>, Sensor 2<sup>+</sup> reporter strain, are elucidated. Production of the signaling molecule by *S. typhimurium*

occurs during growth on preferred carbohydrates that, upon utilization by the bacteria, result in a decrease in the pH of the medium. Lowering the pH of the growth medium in the absence of a preferred carbon source induces limited production of the factor, indicating that the cells are influenced by both the changing pH and the utilization of the carbon source. The signaling activity is degraded by the time the cells reach stationary phase, and protein synthesis is required for degradation of the activity. Osmotic shock following growth on an appropriate carbon source greatly increases the amount of activity present in the *S. typhimurium* culture fluids. This increased activity is apparently due to induction of synthesis of the autoinducer and repression of degradation of the activity. *E. coli* and *S. typhimurium* possess a protein called SdiA which is homologous to LuxR from *V. fischeri* (Wang *et al.*, EMBO J. 10: 3363-3372, 1991; Ahmer *et al.*, J. Bacteriol. 180: 1185-1193, 1998). SdiA is proposed to respond to an extracellular factor (Sitnikov *et al.*, Proc. Natl. Acad. Sci. USA 93: 336-341, 1996; Garcia-Lara *et al.*, J. Bacteriol. 178: 2742-2748, 1996), and it has been shown to control virulence factor production in *S. typhimurium* (Ahmer *et al.*, 1998, *supra*). The analysis set forth below shows that the AI-2 autoinducer signaling activity does not function through the SdiA pathway.

**[0232] *S. typhimurium* LT2 Produces an Autoinducer-like Activity.** Example 1 demonstrates that *S. typhimurium* and *E. coli* strains produce a signaling activity that stimulates *lux* expression in *V. harveyi*, and the signaling molecule acts exclusively through the *V. harveyi* quorum-sensing System 2. Figure 4 shows the induction of luminescence in the *V. harveyi* System 2 reporter strain BB170 (Sensor 1<sup>-</sup>, Sensor 2<sup>+</sup>). The control experiment shows the characteristic quorum-sensing behavior of *V. harveyi* (closed circles). Immediately after dilution into fresh medium, the light emitted per cell by *V. harveyi* drops rapidly, over 1000-fold. At a critical cell density, which corresponds to the accumulation of a critical concentration of endogenously produced autoinducer (AI-2) in the medium, the luminescence per cell increases exponentially, approximately three orders of magnitude, to reattain the pre-dilution level.

**[0233]** Addition of 10% cell-free culture fluid prepared from *V. harveyi* BB152 (AI-1<sup>-</sup>, AI-2<sup>+</sup>) caused the reporter strain to maintain a high level of light output following dilution (open circles). The increased light output is due to the *V. harveyi* BB170 cells

responding to the presence of AI-2 in the cell-free culture fluids prepared from *V. harveyi* strain BB152 (Bassler *et al.*, 1993, *supra*). Similarly, addition of cell-free culture fluid from *S. typhimurium* LT2 grown in LB + 0.5% glucose induced luminescence in the reporter strain approximately 800-fold over the control level (solid squares). No activity similar to *V. harveyi* AI-1 was produced by *S. typhimurium* LT2 under these conditions and there is no AI-1 or AI-2 activity in LB + 0.5% glucose (see Example 1).

**[0234] Environmental Factors Influence Autoinducer Production and Degradation in *S. typhimurium*.** Control of autoinducer production in *S. typhimurium* is different than in other described quorum-sensing systems. Figure 5A demonstrates three important aspects of the regulation of autoinducer production in *S. typhimurium*. First, no autoinducer activity is observed when *S. typhimurium* is grown for 6 h in LB in the absence of glucose. Second, growth in the presence of glucose for 6 h results in substantial production of autoinducer (760-fold activation of the reporter strain). Third, activity, while detectable, is severely reduced when the *S. typhimurium* culture is allowed to grow to stationary phase (33-fold activation of the reporter strain).

**[0235]** We subjected *S. typhimurium* LT2 to several different treatments including some environmental stresses in order to begin to understand what conditions favor autoinducer production versus those that favor autoinducer degradation. In the experiment presented in Figure 5B, the *S. typhimurium* cells were induced for signal production by pre-growth in LB containing 0.5% glucose for 6 h. We have shown that under these conditions, the glucose is not depleted (Surette and Bassler, 1998). After the induction phase of growth, the culture fluid was removed and aliquots of the cells were resuspended and incubated for 2 h under a variety of conditions that are described in the description of Figure 2. Following each of these treatments cell-free fluids were prepared and tested for activity on BB170.

**[0236]** It is important to note that in the results presented in Figure 5B, the *S. typhimurium* were pre-induced for autoinducer production at the start of the experiment, *i.e.*, their cell-free culture fluid activated the reporter strain 760-fold. Figure 5B shows that removal of the pre-growth culture fluid from these cells and resuspension of the cells in LB without glucose, in 0.1 M NaCl (hypotonic conditions), or heat shock at 43°C for 2 h resulted in no or very low autoinducer production. These results indicate that the above treatments



result in termination of autoinducer production, or degradation of newly released autoinducer, or both.

[0237] In contrast to the above results, resuspension of pre-induced cells in fresh LB + glucose resulted in continued high-level production of autoinducer (735-fold activation of the reporter). Similarly, acidic pH promoted continued production of autoinducer (600-fold activation), and hypertonic osmotic shock (0.4 M NaCl) resulted in 1300-fold induction of the reporter. Increased AI-2 activity was only observed in the pH 5.0 fluids or 0.4 M NaCl osmotic shock fluids of cells that were already actively producing AI-2, *i.e.*, if glucose was not included during the pre-growth, no measurable activity was produced following the identical 2 h treatments.

[0238] Shifting *S. typhimurium* cells from LB + glucose to 0.4 M NaCl resulted in an accumulation of AI-2 activity to a level much greater than that observed under any other condition tested. Below it is shown that *S. typhimurium* cells resuspended in 0.4 M NaCl increase the biosynthesis and/or release of autoinducer, and furthermore they apparently do not degrade significant quantities of the released activity. A similar increase in AI-2 production occurs when the *S. typhimurium* cells are resuspended in 0.4 M NaCl, 0.4 M KCl or 0.8M sucrose, indicating that the NaCl effect on AI-2 production is an osmotic one, not an ionic one. This apparent osmotic shock effect on the *S. typhimurium* cells was extremely useful because it enabled us to measure maximal release of autoinducer activity in the absence of loss due to degradation.

[0239] **The Effect of Glucose on Signal Production in *S. typhimurium*.** In Example 1 we showed that the continued presence of glucose was required for *S. typhimurium* to produce the quorum-sensing signaling factor. Because sugar utilization both increases the growth rate while decreasing the pH of the culture, we further analyzed the effect of metabolism of glucose, decreasing pH and increasing cell number on signal production by *S. typhimurium*. In the experiment presented in Figure 6, we measured signal production, growth rate, and pH in growing *S. typhimurium* LT2 cultures containing limiting (0.1%) and non-limiting (1.0%) concentrations of glucose. In the data presented in Figure 6, at various times, the level of autoinducer produced in both the cell-free culture fluids and in the corresponding 0.4 M NaCl osmotic shock fluids was measured and normalized for 1 x

10<sup>9</sup> cells. It should be noted that unlike in Figure 5, the cells in this experiment were not pre-induced for signal production.

[0240] Figure 6 shows that the pattern of production and disappearance of autoinducer observed in 0.4 M NaCl osmotic shock fluids mimics that observed in cell-free culture fluids. However, at every time point that autoinducer is produced, much greater activity is detected in the osmotic shock fluids than in the corresponding cell-free culture fluids. Under conditions of limiting (0.1%) glucose (Figures 6A, 6C and 6E), *S. typhimurium* produces the signaling activity between 2-4 h (Bars). However, the glucose becomes completely depleted at 4 h, and at that time production of the factor ceases (Figure 6A). In contrast, when the cells are grown in 1.0% glucose (Figures 6B, 6D, and 6F), glucose is present in the medium throughout the entire experiment (Figure 6B). Under these conditions, the cells continue to synthesize activity for 12 hours. Similar to the results shown in Figure 5 and those reported in Example 1, almost no activity was observed in cell-free culture fluids or osmotic shock fluids from stationary phase cells at 24 h regardless of the glucose concentration.

[0241] *S. typhimurium* grows at roughly the same rate in both high and low glucose media during exponential phase. In fact, the *S. typhimurium* culture grown in high glucose medium does not reach the cell density achieved by the *S. typhimurium* grown in the low glucose medium (Figures 6C and 6D). Cell growth is probably inhibited in this culture by the dramatically reduced pH that occurs from increased glucose utilization. These results show that the higher level of activity produced by *S. typhimurium* in the LB containing 1% glucose is not due to higher cell number, but due to induction of signal production caused by glucose metabolism.

[0242] Figures 6E and 6F show the pH of the low and high glucose cultures at each time point. Under conditions of low glucose (Figure 6E), the pH of the culture initially decreases as the cells utilize the glucose. However, simultaneous to the complete depletion of the glucose, the pH begins to rise. In contrast, under conditions of high glucose, the pH of the medium decreases to below pH 5 (Figure 6F). In the experiments presented in Figure 6, both glucose catabolism and decreasing pH occur simultaneously suggesting that either or both of these factors could be responsible for signal production by *S. typhimurium*.

**[0243] Both Glucose Metabolism and Low pH Independently Control Signal Production in *S. typhimurium*.** To distinguish between the contribution from glucose metabolism and that from low pH in signal production by *S. typhimurium*, we compared the activity produced by *S. typhimurium* grown in LB containing 0.5% glucose in a culture in which the pH was maintained at 7.2 (Figure 7A), to that produced by *S. typhimurium* grown in LB without glucose where the pH was maintained at 5.0 (Figure 7B). Again, we measured the signal present in cell-free culture fluids and in 0.4 M NaCl osmotic shock fluids. Similar to the data presented in Figure 3, the level of signal observed in cell-free culture fluids was lower than that observed in the 0.4 M osmotic shock fluids.

**[0244]** When *S. typhimurium* was grown in LB + 0.5% glucose at pH 7.2 increasing amounts of the quorum-sensing signal were detected for 6 h. At 6 h, in 0.4 M NaCl osmotic shock fluids, there was an approximately 550-fold stimulation of light production of the *V. harveyi* reporter strain BB170. No activity was produced after the 6 h time point. Figure 7A shows that the pH was maintained between 7.15 and 7.25 for 8 h, after this time, the pH of the culture no longer decreased, but began increasing presumably because the cells had depleted the glucose. We allowed the pH to continue to increase for the duration of the experiment. Also shown in the figure is the cell number at each time point. At pH 7.2, the cells grew rapidly and reached a high cell density.

**[0245]** Analysis of time courses similar to those presented here, has shown that *S. typhimurium* does not produce any signal when it is grown in LB without glucose at neutral pH (see Example 1). However, *S. typhimurium* did transiently produce the quorum-sensing factor in the absence of glucose when grown at pH 5.0 (Figure 7B). Signal was produced for 4 h, and about 450-fold stimulation of the reporter was the maximum activity achieved in 0.4 M NaCl osmotic shock fluids. Very little signal was produced by 5 h, and signal was completely absent after 6 h of incubation. Figure 7B shows that the pH was maintained between 5.0 and 5.2 in this experiment. Note that the cells grew much more slowly at pH 5.0 than at pH 7.2.

**[0246] Preliminary Characterization of the *S. typhimurium* Autoinducer Degradative Apparatus.** The quorum-sensing activity produced by *S. typhimurium* LT2 is degraded by the onset of stationary phase. We have determined that the activity contained in

cell-free culture supernatants and 0.4 M NaCl osmotic shock fluids from cells grown for 6 h in LB + glucose is stable for at least 24 h at 30°C, indicating that no degradative activity is present in these cell-free fluids. Furthermore, mixing cell-free culture fluids prepared from actively producing *S. typhimurium* (*i.e.*, from cultures grown for 6 h in LB + glucose) with cell-free culture fluids prepared from *S. typhimurium* that have degraded the factor (*i.e.*, from cultures grown for 12 or 24 h in LB + glucose) does not result in degradation of the activity. This result indicates that the degradative activity is not released, but instead, is associated with the cells.

[0247] We show in Figure 5 that no further autoinducer is produced if *S. typhimurium* cells that are actively releasing autoinducer are shifted to 0.1M NaCl. However, when these same cells are shifted to 0.4 M NaCl, we observe even greater autoinducer production. This result implies that low osmolarity could be a signal that induces the autoinducer degradative machinery. To begin to analyze the mechanism by which osmolarity affects autoinducer production and degradation in *S. typhimurium*, we investigated the requirement for protein synthesis in signal production and degradation by *S. typhimurium* in high and low osmolarity. As described in the legend to Figure 5, *S. typhimurium* LT2 was grown in LB containing 0.5% glucose to achieve maximal induction of signal production then treated with 0.1M or 0.4 M NaCl in the presence and absence of protein synthesis. Cell-free fluids were prepared and tested for signaling activity. Because half of the cell-free osmotic shock fluids contained chloramphenicol (Cm), *V. harveyi* JAF305 was used as the reporter strain in the activity assay. This *V. harveyi* strain contains a Cm<sup>r</sup> cassette in the *luxN* gene, and its phenotype is Sensor 1<sup>-</sup>, Sensor 2<sup>+</sup>, a phenotype identical to that of *V. harveyi* BB170.

[0248] When the cells were resuspended in 0.4 M NaCl, the *S. typhimurium* produced and released increasing amounts of the signal for 200 min (Figure 8A, open squares). After this time, the level of signaling activity present in the cell-free osmotic shock fluid decreased somewhat, suggesting that some of the released signal was degraded. Quite different results were obtained when the *S. typhimurium* cells were resuspended in 0.1M NaCl (Figure 8B, open squares). In this case, at early time points, the *S. typhimurium* produced a quantity of activity equivalent to that produced by cells resuspended in 0.4 M

NaCl. However, by 120 min, no activity remained in the cell-free low osmolarity fluid. This result indicates that under conditions of low osmolarity, the released activity is rapidly degraded. We do not observe degradation of the activity in cell-free culture fluids, indicating that the disappearance of the activity from low osmolarity cell-free fluids is not due to chemical instability of the signaling molecule.

[0249] Under conditions of high osmolarity, when the cells were treated with Cm to inhibit protein synthesis, only about one quarter of the activity was produced compared to untreated cells. The closed squares in Figure 8A show that 300-fold induction of the reporter strain occurred in the presence of Cm as compared to 1200-fold induction with the untreated cells (Figure 8A, open squares). When the *S. typhimurium* was resuspended in low osmolarity (Figure 8B), roughly three-quarters of the activity produced in the absence of Cm (open squares) was produced in the presence of Cm (closed squares). In the presence of Cm, the released activity was not degraded by 300 min in high osmolarity and only partially degraded in low osmolarity.

[0250] To show that high osmolarity does not inhibit AI-2 signal degradation, we added the activity contained in the 0.4 M NaCl cell-free osmotic shock fluids to *S. typhimurium* cells that had been resuspended in 0.1M NaCl for two hours. As shown in Figure 8, these are cells that can degrade the factor. Table 3 shows that these *S. typhimurium* cells degraded greater than 98% of the signaling activity while incubated at high osmolarity. The table also shows that *S. typhimurium* cells that had been incubated in 0.4 M NaCl (these are cells that are actively producing the signal) released no further activity when resuspended in the 0.1M NaCl incubation fluid obtained from the actively degrading cells. Furthermore, mixing active and inactive 0.4 M and 0.1M cell-free osmotic fluids did not result in degradation of the activity in the 0.4 M fluids.

Table 3

High osmolarity induces release and low osmolarity induces degradation  
of the *S. typhimurium* signaling factor

Treatment	Fold-induction of Luminescence
0.1M NaCl activity <sup>a</sup>	4
0.1M NaCl activity <sup>a</sup>	4
0.4 M NaCl activity <sup>a</sup>	944
0.1M cells + 0.4 M activity <sup>b</sup>	17
0.4 M cells + 0.1M activity <sup>c</sup>	6

<sup>a</sup>*S. typhimurium* was grown for 6 h in LB containing 0.5% glucose. The cells were pelleted and resuspended in either 0.1M or 0.4 M NaCl for 2 h. Cell-free fluids were prepared and tested for activity.

<sup>b</sup>*S. typhimurium* cells that had been incubated in 0.1M NaCl for two hours were pelleted and resuspended in the activity contained in the cleared osmotic shock fluids obtained from cells suspended in 0.4 M NaCl for 2 h. Cell-free fluids were prepared after a 2 h incubation and assayed for signaling activity.

<sup>c</sup> *S. typhimurium* cells that had been suspended in 0.4 M NaCl were pelleted and incubated for 2 h in the cleared osmotic shock fluids obtained from cells suspended for 2 h in 0.1M NaCl. Cell-free fluids were prepared after the 2 h incubation and assayed for signaling activity.

**[0251] The LuxR Homolog SdiA is not Involved in Response to the AI-2 Autoinducer.** A gene homologous to *luxR* of *V. fischeri* has been identified in *E. coli* and *S. typhimurium* and is called *sdiA*. Two reports suggest that in *E. coli*, SdiA modestly regulates the expression of the cell division locus *ftsQAZ* in response to a factor present in cell-free culture fluids (Garcia-Lara *et al.*, 1996, *supra*), and in response to a few homoserine lactone autoinducers (Sitnikov *et al.*, 1996, *supra*). Completion of the sequence of the *E. coli* genome shows that no LuxI homologue exists in *E. coli* so the locus responsible for the biosynthesis of the hypothesized soluble factor(s) has not been determined. Overexpression of SdiA in *S. typhimurium* has recently been shown to influence the expression of several ORFs located on the *S. typhimurium* virulence plasmid (Ahmer *et al.*, 1998, *supra*). As in the *E. coli* studies, SdiA activity in *S. typhimurium* is proposed to be regulated by an extracellular factor.

[0252] It was possible that the AI-2 autoinducer that we have been characterizing in *S. typhimurium* and *E. coli* acted through SdiA. We tested whether AI-2 had an effect on genes regulated by SdiA in *E. coli* and *S. typhimurium*. In *E. coli*, we assayed an *ftsQ1p2p-lacZ* reporter, and in *S. typhimurium* we assayed an *rck::MudJ* fusion in both an *sdiA*<sup>+</sup> and *sdiA*<sup>-</sup> background. We tested the effects of addition of LB, 0.4 M NaCl, 0.4 M NaCl osmotic shock fluids containing AI-2 activity from *S. typhimurium* LT2, *E. coli* O157, and 0.4 M NaCl osmotic shock fluid from *E. coli* DH5 $\alpha$ . We have shown previously in Example 1 that DH5 $\alpha$  does not produce AI-2 activity under our growth conditions.

[0253] For the *E. coli* experiments we determined that MC4100 and MC4100/pMS209 (containing *ftsQ1p2p* in the incorrect orientation) had no measurable-galactosidase activity. The level of -galactosidase produced by MC4100/pMS207 (containing the *ftsQ1p2p-lacZ* fusion) was roughly 20-30 Miller units, and this level of activity did not change under any of the conditions tested here. This level of activity of the fusion was comparable to that reported previously (Sitnikov *et al.*, 1996, *supra*; Garcia-Lara *et al.*, 1996, *supra*). In the *S. typhimurium* SdiA studies, similar to Ahmer *et al.* (1998, *supra*), we obtained ~30 Miller units of *rck::MudJ* activity in the *sdiA*<sup>+</sup> background and this level was reduced to 10 units in the *sdiA*<sup>-</sup> background. No change in -galactosidase production occurred following the addition of AI-2 from *E. coli* or *S. typhimurium*. These results indicate that, if an extracellular factor exists that regulates the activity of SdiA, under the conditions we have tested, it is not AI-2.

[0254] **Quorum Sensing in *E. coli* and *S. typhimurium*.** We have developed a heterologous bio-assay that enables detection of an extracellular signaling factor produced by *S. typhimurium*. The factor mimics the action of AI-2 of the quorum-sensing bacterium *V. harveyi*, and it acts specifically through the *V. harveyi* Signaling System 2 detector LuxQ. Results using *lacZ* fusions to the *ftsQ* and *rck* promoters indicate that, under our assay conditions, the AI-2 quorum-sensing factor does not signal to SdiA, at least with respect to regulation of these genes. The AI-2 quorum-sensing system is therefore involved in a different *S. typhimurium* and *E. coli* signal transduction pathway than the one(s) investigated previously.

[0255] *S. typhimurium* LT2 produces an amount of activity roughly equivalent to that produced by *V. harvey*, with approximately 800-fold stimulation of the *V. harveyi* reporter strain BB170 upon addition of 10% *S. typhimurium* cell-free culture fluids. The timing of *lux* induction and the shape of the response curve of *V. harveyi* to the *S. typhimurium* signal are indistinguishable from those of *V. harveyi* responding to its own AI-2. Furthermore, we have been successful at partially purifying both the *V. harveyi* AI-2 and the *S. typhimurium* signal molecule using identical purification procedures. These two results lead us to believe that the *S. typhimurium* signaling molecule is identical to or very closely related to AI-2 of *V. harveyi*.

[0256] **Growth Conditions Regulate Signal Production and Degradation in *S. typhimurium*.** In this example, we further characterize the regulation of the signaling activity in *S. typhimurium* LT2. Accumulation of signaling activity in *S. typhimurium* culture supernatants is maximal during mid-exponential phase when the cells are actively using glucose in rich medium. Under these growth conditions, use of glucose is accompanied by a rapid drop in pH of the culture. The results demonstrate that either glucose metabolism or low pH induces *S. typhimurium* LT2 to produce the quorum-sensing factor, indicating that both glucose and acidity generate independent signals for autoinducer production. In the presence of glucose, when the pH is not maintained, probably both the decreasing pH and the presence of an appropriate carbon source contribute to the regulation of quorum sensing in *S. typhimurium*. The results also show that production of the autoinducer ceases before stationary phase in the presence of glucose at neutral pH and in the absence of glucose at low pH. Therefore, a combination of acidic conditions and the absence of glucose is not required to cue *S. typhimurium* to terminate production of autoinducer.

[0257] In addition to glucose, growth on several other carbohydrates also induces production of the signaling activity. These include both PTS (fructose, mannose, glucitol, and glucosamine) and non-PTS (galactose and arabinose) sugars. These findings eliminate an exclusive role for the PTS in the regulation of autoinducer biosynthesis. When the *S. typhimurium* LT2 are grown on several other carbon sources (acetate, glycerol, citrate and serine) no significant accumulation of signaling activity is observed. Example 1 shows that the signal is not any of a number of substances known to be secreted by *S. typhimurium*,



including the major products of mixed acid fermentation. Clearly, the cells precisely regulate production of the signaling molecule and favor its production when growing on preferred carbohydrates. Identification of the signaling molecule and cloning of the biosynthetic gene(s) will aid in a fuller understanding of the regulation process.

**[0258]** This example shows that, in contrast to other quorum-sensing systems, the *S. typhimurium* signal does not accumulate in stationary phase. Autoinducer production and degradation both contribute to this regulation. This example establishes autoinducer production as an increase in the signaling activity present in cell-free fluids. Activity could increase from release of newly biosynthesized autoinducer, release of stored autoinducer, repression of degradation of autoinducer, or some combination of these. We define autoinducer degradation as the disappearance of signaling activity from the cell-free fluids. This disappearance could be due to destruction of the autoinducer, re-uptake of the autoinducer, or a combination of these activities. Autoinducer production and degradation could occur simultaneously under some conditions. These findings indicate that quorum sensing in *S. typhimurium* is regulated such that the signal and presumably the response to the signal do not persist into stationary phase. Because signal production requires use of a preferred carbohydrate, quorum sensing in *S. typhimurium* may be used for measuring both the cell density and the potential of the environment for growth.

**[0259] Osmolarity Influences Signal Production and Degradation in *S. typhimurium*.** *S. typhimurium* cells that are actively producing signal can be further stimulated to produce signal by specific environmental treatments, such as 0.4 M NaCl osmotic shock, which indicates that several independent regulatory pathways channel information into autoinducer synthesis. On resuspension in 0.4 M NaCl, *S. typhimurium* cells producing autoinducer exhibit significantly greater activity when they can synthesize protein than when protein synthesis is blocked. Furthermore, degradation of the signal also requires protein synthesis.

**[0260]** These results have several implications. First, in the presence of Cm, *S. typhimurium* resuspended at both high and low osmolarity produce a similar amount of activity. This result indicates that, following growth in the presence of glucose, the *S. typhimurium* cells have a pre-defined capacity to produce signaling activity (and/or to release

already synthesized activity from the cell). Second, resuspension of the cells in high osmolarity medium increases signal production well beyond this level, which requires protein synthesis. High osmolarity is apparently one environmental cue that induces *S. typhimurium* to synthesize more of the biosynthetic apparatus necessary for signal production and/or release. Third, low osmolarity causes an initial release of activity, followed by a rapid degradation that requires protein synthesis because it does occur in the presence of Cm. These results imply that the environment has changed from conditions favoring autoinducer production (LB + a preferred carbohydrate, or high osmolarity) to conditions where autoinducer production is not favored (low osmolarity, or absence of a preferred carbon source). This environmental change induces *S. typhimurium* to synthesize the protein(s) required for degradation of the signaling activity.

[0261] When the *S. typhimurium* cells were incubated in 0.4 M NaCl no significant degradation of the activity occurred by 200 min. This result indicates that either the necessary degradative protein(s) are not synthesized under these conditions, or alternatively, the degradative apparatus is assembled, but its activity is inhibited by high osmolarity. The results show that high osmolarity does not inhibit signal degradation, because cells induced to degrade the activity can do so at high osmolarity. Therefore, the persistence of the activity in the high NaCl samples occurs because the degradation machinery is not synthesized, not because its activity is inhibited.

[0262] It is difficult to determine precisely when *S. typhimurium* cells produce autoinducer and when they degrade it because both processes could occur simultaneously. It appears, however, that little or no degradation occurs in high osmolarity, that conversion of cells from overall signal producers to overall signal degraders occurs in low osmolarity and that degradation requires protein synthesis. Preliminary characterization indicates that degradation is cell-associated, because the autoinducer activity is stable in cell-free culture supernatants for long periods. In addition, combining active with inactive cell-free culture fluids or active and inactive high and low osmolarity cell-free fluids does not promote degradation of the autoinducer.

[0263] **The Role for Quorum Sensing in *Salmonella* Pathogenesis.** The observations presented here on the regulation of signal production and degradation by *S.*

*typhimurium* LT2 implicate a role for quorum sensing in pathogenesis of *Salmonella*. The conditions favoring signal production (nutrient rich, high osmolarity and low pH) are those likely to be encountered upon the first interaction of an enteric pathogen with its host. Conditions favoring degradation of the signal (nutrient poor, low osmolarity) are those most probably encountered as the pathogen exits the host. The initial colonization of the host may be a concerted effort between a population of cells coordinated through this cell-cell signaling system. Other cues, that we have not yet tested, could also regulate quorum sensing in *S. typhimurium*. These may represent independent or overlapping signaling pathways involved in pathogenesis. We are isolating *S. typhimurium* mutants to test these hypotheses. Finally, *Salmonella* pathogenesis is a dynamic process of interaction between the host and metabolically active bacteria. Consistent with a role for quorum sensing in pathogenesis, our evidence suggests that this quorum-sensing system is not functioning during stationary phase. We have shown that the signaling molecule is not produced during stationary phase, and furthermore, existing signal is degraded. Perhaps quorum sensing is critical for *S. typhimurium* to undergo the transition between a host-associated and a free-living existence.

### EXAMPLE 3.- Quorum Sensing in Escherichia coli, Salmonella typhimurium and Vibrio harveyi: A New Family of Genes Responsible for Autoinducer Production

[0264] Genes responsible for AI-2 production in *V. harveyi*, *E. coli* and *S. typhimurium* (named *luxS<sub>V.h.</sub>*, *luxS<sub>E.c.</sub>*, and *luxS<sub>S.t.</sub>* respectively) are highly homologous to each other, and are thought to define a new family of proteins involved in autoinducer production. The genes have been identified in many bacteria by genome sequencing projects, but until now no function has been ascribed to them in any organism. The *luxS* genes do not bear homology to any other gene known to be involved in autoinducer production.

[0265] **Identification and Cloning of the Gene Responsible for AI-2 Production in *V. harveyi*.** Previous examples show that, unlike many other *E. coli* strains, *E. coli* strain DH5 $\alpha$  does not produce an AI-2 signal molecule that can be detected by *V. harveyi*. We reasoned therefore, that we could use *E. coli* DH5 $\alpha$  as a mutant to clone the *V. harveyi* AI-2 production gene. A library of wild type *V. harveyi* BB120 genomic DNA was transformed into *E. coli* strain DH5 $\alpha$ , and the transformants were screened for AI-2

production in the *V. harveyi* BB170 AI-2 detection bioassay. The library consisted of 2,500 clones each containing roughly 25 kb of *V. harveyi* genomic DNA. Five DH5 $\alpha$  clones were identified that resulted in upwards of 300-fold stimulation of the reporter strain in the bioassay.

[0266] The recombinant cosmid DNA from the five AI-2 producing *E. coli* DH5 $\alpha$  clones was analyzed by restriction analysis and Southern blotting. All five of the cosmids contained an overlapping subset of identical *V. harveyi* genomic restriction fragments, indicating that we had cloned the same locus several times. One cosmid, called pBB2929 was selected for further analysis. Random mutagenesis using transposon Tn5 was carried out on cosmid pBB2929, and pools of cosmids harboring Tn5 insertions were subsequently transformed into *E. coli* DH5 $\alpha$ . We tested 962 individual *E. coli* DH5 $\alpha$ /pBB2929::Tn5 strains for the loss of the ability to produce AI-2. Four *E. coli* DH5 $\alpha$  strains harboring Tn5 insertions in pBB2929 were identified that failed to produce AI-2. We mapped the locations of these Tn5 insertions in pBB2929 and found that all four transposon insertions resided in the same 2.6 kb *Hind*III *V. harveyi* genomic DNA fragment (Figure 9A).

[0267] Cosmid pBB2929 was digested with *Hind*III and the 8 resulting fragments were subcloned in both orientations into pALTER (Promega). The pALTER subclones were transformed into *E. coli* DH5 $\alpha$ , and subsequently tested for AI-2 production. The only strains capable of producing AI-2 contained the 2.6 kb *Hind*III fragment identified in the Tn5 mutagenesis. This fragment was sequenced, and only one open reading frame (ORF) could be identified, and its location corresponded to the map positions of the four Tn5 insertions that eliminated AI-2 production. We named the ORF LuxS<sub>*V.h.*</sub> (Figure 9A).

[0268] **Mutagenesis of luxS<sub>*V.h.*</sub> in *V. harveyi*.** We analyzed the effects of luxS<sub>*V.h.*</sub> null mutations on AI-2 production in *V. harveyi*. The four Tn5 insertions that mapped to the LuxS<sub>*V.h.*</sub> gene and the control Tn5 insertion adjacent to the luxS<sub>*V.h.*</sub> locus were transferred to the corresponding locations in the *V. harveyi* BB120 chromosome to make strains MM37, MM30, MM36, MM38 and MM28, respectively (Figure 9A). Southern blotting was used to confirm the correct placement of all five Tn5 insertions in the *V. harveyi* chromosome. The four *V. harveyi* luxS<sub>*V.h.*</sub>::Tn5 insertion strains were tested for the ability to produce AI-2, and all four strains gave identical results.

[0269] In Figure 10A, we show the AI-2 production phenotypes of the wild type control Tn5 insertion strain MM28 and one representative *luxS<sub>V.h.</sub>::Tn5* insertion strain, MM30. *V. harveyi* MM28 and MM30 were grown to high cell density, after which cell-free culture fluids were prepared. The culture fluids were assayed for AI-2 activity by the ability to induce luminescence in the AI-2 detector strain BB170. Figure 10A shows that addition of culture fluids from the control Tn5 insertion strain MM28 induced luminescence in the reporter 780-fold, while culture fluid from the *luxS<sub>V.h.</sub>::Tn5* insertion strain MM30 did not induce the expression of luminescence in the reporter. Therefore, a null mutation in *luxS<sub>V.h.</sub>* in *V. harveyi* eliminates AI-2 production.

[0270] **Identification and Analysis of *S. typhimurium* Autoinducer Production Mutants.** To identify the gene responsible for AI-2 production in *S. typhimurium*, we randomly mutagenized *S. typhimurium* LT2 using the MudJ transposon (Maloy *et al.*, 1996, *supra*). Ten-thousand *S. typhimurium* LT2 insertion mutants were assayed for AI-2 production in the *V. harveyi* BB170 bioassay. One *S. typhimurium* MudJ insertion mutant (strain CS132) was identified that lacked detectable AI-2 in culture fluids at mid-exponential phase.

[0271] Figure 10B shows the AI-2 production phenotypes of *S. typhimurium* strain LT2 and the corresponding MudJ insertion strain CS132. The strains were grown to mid-exponential phase in LB containing glucose, and cell-free culture fluids were prepared and assayed for AI-2. *S. typhimurium* LT2 culture fluids induced the reporter strain 500-fold, while culture fluids from strain CS132 contained no AI-2 activity. Furthermore, strain CS132 did not produce AI-2 under any of the growth conditions that we have previously reported induce AI-2 production in *S. typhimurium* (not shown).

[0272] The site of the MudJ insertion in *S. typhimurium* CS132 was determined by PCR amplification followed by sequencing of the 110 bp of chromosomal DNA adjacent to the transposon. This sequence was used to search the database for DNA homologies. The sequence matched a site (89/105 bp identity) in the *E. coli* MG1655 genome that corresponded to an open reading frame of unknown function denoted *ygaG* (Blattner *et al.*, 1997, *supra*). In the chromosome, the *E. coli ygaG* gene is flanked by the *gshA* and *emrB* genes (Figure 9B). The *ygaG* gene is transcribed from its own promoter which is located

immediately upstream of the gene, indicating that it is not in an operon with *gshA*. The *emrB* gene is transcribed in the opposite direction. We PCR amplified the *ygaG* region from the chromosomes of *E. coli* O157:H7 and *E. coli* MG1655, and the two *E. coli ygaG* genes were cloned into pUC19.

**[0273] Complementation of *S. typhimurium* and *E. coli* AI-2<sup>-</sup> Mutants.** We tested whether the *E. coli* O157:H7 *ygaG* gene and the *V. harveyi luxS<sub>V.h.</sub>* gene could restore AI-2 production in the AI-2<sup>-</sup> strains *S. typhimurium* CS132 and *E. coli* DH5 $\alpha$ . In Figure 11A, we show the AI-2 activity produced by wild type *V. harveyi* BB120, *E. coli* O157:H7 and *S. typhimurium* LT2. In this figure, the level of AI-2 activity present in *V. harveyi* BB120 cell-free culture fluids was normalized to 100%, and the activities in cell-free culture fluids from *E. coli* and *S. typhimurium* compared to that. In this experiment, *E. coli* O157:H7 produced 1.5 times and *S. typhimurium* LT2 produced 1.4 times more AI-2 activity than *V. harveyi* BB120 (*i.e.*, 150% and 141%, respectively).

**[0274]** Figure 11B and 11C show the AI-2 complementation results for *S. typhimurium* CS132 and *E. coli* DH5 $\alpha$ . Figure 11B demonstrates that introduction of the *E. coli* O157:H7 *ygaG* gene into *S. typhimurium* CS132 restored AI-2 production beyond the level of production of wild type *S. typhimurium* (*i.e.*, 209% activity). Comparison of the data in Figure 11A and 11B shows that the *E. coli ygaG* gene in *S. typhimurium* resulted in AI-2 production exceeding that produced *in vivo* by *E. coli* O157:H7. Introduction of the *V. harveyi* LuxS<sub>V.h.</sub> gene into *S. typhimurium* resulted in AI-2 production at slightly less than the level produced by wild type *V. harveyi* BB120 (*i.e.*, 73% of the level of *V. harveyi* BB120). Figure 11C shows that *E. coli* DH5 $\alpha$  was also complemented to AI-2 production by both the cloned *E. coli* O157:H7 and the *V. harveyi* BB120 AI-2 production genes. However, introduction of *E. coli* O157:H7 *ygaG* and *V. harveyi* BB120 *luxS<sub>V.h.</sub>* into *E. coli* DH5 $\alpha$  resulted in only 31% and 43% of the *V. harveyi* BB120 AI-2 activity, respectively. Figure 11B and 11C show that the control vectors produced no activity in the complementation experiments.

**[0275] Analysis of the AI-2 Production Genes from *V. harveyi*, *E. coli* and *S. typhimurium*.** We sequenced the AI-2 production gene LuxS<sub>V.h.</sub> from *V. harveyi* BB120 and the *ygaG* loci from *E. coli* O157:H7, *E. coli* MG1655 and *E. coli* DH5 $\alpha$ . The translated

protein sequences encoded by the *ygaG* ORF's are shown in Figure 12, and they are aligned with the translated LuxS protein sequence from *V. harveyi*. The non-bold, underlined amino acids indicate the residues in the *E. coli* proteins that differ from the *V. harveyi* LuxS protein. The *ygaG* loci from *E. coli* encode proteins that are highly homologous to one another and also to LuxS from *V. harveyi*. The *E. coli* MG1655 (SEQ ID NO: 25) and the *E. coli* O157:H7 (SEQ ID NO: 11) YgaG proteins are 77% and 76% identical to LuxS from *V. harveyi* BB120 (SEQ ID NO: 10). The DNA sequence we determined for *ygaG* from *E. coli* O157:H7 differs at five sites from the reported (and our) sequence for the *E. coli* MG1655 *ygaG* gene. Four of the changes are silent, the fifth results in a conservative Ala to Val alteration at amino acid residue 103 in the *E. coli* O157:H7 protein.

[0276] Identification of the *ygaG* locus in *E. coli* MG1655 and *E. coli* O157:H7 allowed us to investigate the AI-2 production defect in *E. coli* DH5 $\alpha$ . *E. coli* DH5 $\alpha$  possesses the *ygaG* gene because we could PCR amplify this region from the chromosome using the same primers we employed to amplify it from *E. coli* MG1655 and *E. coli* O157:H7. Examination of the *E. coli* DH5 $\alpha$  *ygaG* promoter showed that it is identical to that of *E. coli* MG1655, indicating that the AI-2 defect in *E. coli* DH5 $\alpha$  is not simply due to decreased transcription of *ygaG*. However, sequence analysis of the *E. coli* DH5 $\alpha$  *ygaG* coding region showed that a one G-C base pair deletion and a T to A transversion exist at bp 222 and 224, respectively. The frameshift mutation resulting from the G/C deletion causes premature truncation of the *E. coli* DH5 $\alpha$  protein. Figure 12 shows that the truncated *E. coli* DH5 $\alpha$  protein is 111 amino acids, while the *E. coli* MG1655 and *E. coli* O157:H7 proteins are 171 residues. Twenty altered amino acids are translated after the frame shift and prior to termination of the protein. Our complementation results (Figure 11) demonstrate that the AI-2 production defect in *E. coli* DH5 $\alpha$  is recessive to *in trans* expression of *ygaG*, which is consistent with the defect being due to a null mutation caused by the frame shift in the *E. coli* DH5 $\alpha$  *ygaG* gene.

[0277] We searched the *S. typhimurium* database using the sequence we obtained adjacent to the MudJ that inactivated the AI-2 production function in *S. typhimurium* CS132. A perfect match (110/110 bp) was identified to fragment B\_TR7095.85-T7 in the *S. typhimurium* LT2 genome-sequencing database (Genome Sequencing Center, Washington

University, St. Louis). However, the *S. typhimurium* LT2 database *ygaG* sequence (SEQ ID NO: 26) is incomplete (Figure 12). The translated sequence matches the *E. coli* and *V. harveyi* sequences beginning at amino acid residue 8. The translated sequence shows that the *S. typhimurium* protein is 75% identical to LuxS of *V. harveyi*. In order to align the *S. typhimurium* sequence with the *V. harveyi* LuxS protein, we corrected three apparent frame shift errors in the database sequence. Considering that only crude, unannotated sequence data is currently available for *S. typhimurium*, we predict that the *S. typhimurium* protein contains seven more amino acids, and that the frame shift mutations are sequencing errors. We were unsuccessful at PCR amplifying either the *S. typhimurium* 14028 or the *S. typhimurium* LT2 *ygaG* gene using the primers designed for *E. coli*, so we do not yet have the complete sequence of the *S. typhimurium* gene.

[0278] The results set forth above indicate that the genes we have identified and analyzed encode a novel family of proteins responsible for autoinducer production. Members of this new family of genes, referred to herein as LuxS, are highly homologous to one another but not to any other identified genes. The encoded product of the LuxS genes catalyze an essential step in the synthesis of the signaling molecules of the present invention.

#### EXAMPLE 4 - Construction of a Sensor 1<sup>-</sup>, AI2<sup>-</sup> *V. harveyi* Reporter Strain

[0279] *V. harveyi* null mutants in each of the *lux* genes *luxL*, *luxM*, *luxN*, *luxS* and *luxQ* fail either to make or to respond to one specific autoinducer, but they still produce light because, in each case, one quorum-sensing system remains operational. A double *luxN*, *luxS* *V. harveyi* mutant does not emit light without the addition of exogenous AI-2 because this mutant does not respond to AI-1 and it does not produce AI-2.

[0280] The *V. harveyi* LuxS gene has been cloned into *E. coli* DH5α on a broad host range mobilizable cosmid called pLAFR2. This construction restores AI-2 production to *E. coli* DH5α. A marked null mutation was engineered into the *luxS* gene by introducing a chloramphenicol resistance (Cm<sup>r</sup>) cassette into an internal restriction site. Placement of the Cm<sup>r</sup> cassette at this site in *luxS* subsequently eliminated AI-2 production in *E. coli* DH5α.

[0281] The *luxS*::Cm<sup>r</sup> null allele was transferred onto the chromosome of *V. harveyi* strain BB170. Strain BB170 contains a Tn5Kan<sup>r</sup> in *luxN* and does not respond to AI-



1. To construct the double mutant, triparental conjugations were carried out by mixing stationary phase cultures of *E. coli* DH5 $\alpha$  carrying the *V. harveyi luxS::Cm<sup>r</sup>* construction in pLAFR2 (pLAFR2 carries tetracycline resistance), *E. coli* DH5 $\alpha$  carrying the *tra* donor plasmid pRK2013 and the *V. harveyi* recipient strain BB170. Exchange of the *luxS::Cm<sup>r</sup>* mutant allele for the wild type *luxS* allele on the chromosome occurs by homologous recombination. The exogenote cosmid in *V. harveyi* was eliminated by the introduction of a second incompatible plasmid pPH1JI. This was accomplished by mating *E. coli* DH5 $\alpha$  containing pPH1JI with the *V. harveyi* BB170 recipient containing the *luxS::Cm<sup>r</sup>* cosmid, and selecting for exconjugants on plates containing ampicillin (for counter selection of the *E. coli* donor) chloramphenicol (for inheritance of the mutant *luxS::Cm<sup>r</sup>* allele) and gentamicin (for maintenance of the plasmid pPH1JI). Southern blot analysis was used to verify that the exogenote pLAFR2 cosmid has been eliminated and that the *luxS::Cm<sup>r</sup>* construction had been transferred to the corresponding position in the genome of *V. harveyi*. The pPH1JI cosmid was subsequently eliminated by growth in the absence of gentamicin selection.

**[0282] Verification that the LuxN, LuxS Double Mutant Responds to AI-2.**

The *V. harveyi* strain that is mutant in *luxN* and *luxS* was stimulated to produce light in response to the exogenous addition of AI-2 but not AI-1. This was verified in a luminescence assay for response to *V. harveyi* AI-1 and AI-2. *V. harveyi* strain MM30 (*luxS::Tn5*) which is phenotypically AI-1<sup>+</sup>, AI-2<sup>-</sup>, and *V. harveyi* strain BB152 (*luxM::Tn5*) which is phenotypically AI-1<sup>-</sup>, AI-2<sup>+</sup> were used as the sources of AI-1 and AI-2, respectively. The AI-1 and AI-2 present in culture fluids of these strains was tested for stimulation of light production of the *V. harveyi* LuxN, LuxS double mutant reporter strain. In this assay, autoinducer preparations from MM30, BB152 or sterile medium controls were added to the wells of microtiter plates, followed by the addition of the *V. harveyi* reporter strain. The resulting light production was monitored using a liquid scintillation counter in the chemiluminescence mode. Maximal stimulation of light production in the *V. harveyi luxN, luxS* reporter strain was compared to that produced by the Sensor 1<sup>+</sup>, Sensor 2<sup>-</sup> *V. harveyi* strain BB886 and the Sensor 1<sup>-</sup>, Sensor 2<sup>+</sup> *V. harveyi* strain BB170. These two *V. harveyi* strains are routinely used in this assay as reporters of AI-1 and AI-2 activity, respectively.

**[0283] Determine Optimum Concentrations of AI-2 in Microtiter Assays.** The aforementioned screen will be optimized for use in 96-well microtiter assays. The screen will be used in inhibitor assays for identifying inhibitors of AI-2. Purified or synthetic AI-2 will be added to the microtiter wells containing the newly constructed reporter strain and inhibition will be measured by a decrease in light emission from the wells containing an inhibitor. The assay will be optimized by determining the concentration of cells and AI-2 in the microtiter wells that will allow for maximal sensitivity. The optimal AI-2 concentration will be that which stimulates half-maximal light output for a given concentration of cells per unit time. Initial experiments will be conducted in this concentration range to determine the range of AI-2 concentration that produces the greatest change in light output. Similar experiments using AI-1 and a non self-stimulating sensor-1<sup>+</sup>, sensor-2<sup>-</sup> mutant (BB886) showed that the assay was sensitive to concentrations as low as 100nM AI-1 and that light emission was linear over 6 orders of magnitude (light emission from a self-stimulating strain was linear over 3 orders). Similar results for AI-2 using the new reporter strain that will not self-stimulate and therefore have zero background light emission are predicted. Light emission from the microtiter wells will be measured with a Wallac TriLux liquid scintillation counter model 1450-021 in the chemiluminescence mode. This machine will accommodate 16 plates and will therefore allow for 1536 separate concentration experiments per run.

EXAMPLE 5 - In-Vitro Method for Synthesizing AI-2; Purification and Identification of AI-2.

[0284] Several lines of evidence show that autoinducer-2 is not an acyl-homoserine lactone. Autoinducer-2 resists purification by conventional techniques used for the isolation of acyl-homoserine lactone autoinducers such as AI-1 from *V. harveyi*. Unlike other acyl-homoserine lactone autoinducers, AI-2 activity does not extract quantitatively into organic solvents. Furthermore, autoinducer-2 fails to bind to either a cation or anion exchange column. The present characterization of autoinducer-2 indicates that it has a molecular weight of less than 1000 kDa, and is a polar but apparently uncharged organic compound. The AI-2 activity is acid-stable, base-labile, and withstands heating to about 80°C but not 100°C. The *luxS* genes identified bear no homology to other genes known to be involved in production of HSL autoinducers further indicating that the present AI-2 class of autoinducers is novel.

[0285] Thus, in addition to providing a cloned, overexpressed, and purified *S. typhimurium* LuxS protein, the present invention also provides a method for producing AI-2 *in vitro*. The present invention provides a mechanism for generating large quantities of pure AI-2 useful for mass spectral and NMR analysis, and for screening compounds that regulate the activity of AI-2. Moreover, the present invention provides a method for determining the *in vivo* biosynthetic pathway for AI-2 synthesis.

[0286] Analysis of the genomic locations of the various *luxS* genes identified in the present invention indicates that the *luxS* genes do not consistently reside in any one location in the chromosome, nor do they typically appear in close proximity to any specific gene(s). In one case, however, the *luxS* gene is the third gene in a three-gene operon with two genes (*metK* and *pfs*). In *E. coli*, *Salmonella* and many other bacteria, MetK and Pfs are involved in the conversion of S-adenosyl methionine (SAM) to homocysteine and autoinducer-2 (Figure 15). MetK converts methionine to SAM, which is an important cofactor in one-carbon metabolism. SAM methylates DNA, RNA, and a variety of cell proteins, and several SAM dependent methyl transferases act at this step. S-adenosyl homocysteine (SAH) results from transfer of the methyl group from SAM to its substrates. SAH strongly inhibits SAM dependent methyltransferases. Therefore, bacteria rapidly degrade SAH via the enzyme Pfs. The designation “pfs” refers to an open reading frame in the *E. coli* genome that has recently been determined to encode the enzyme 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase, also known as MTA/SAH

nucleosidase. In the present system, the enzyme cleaves the glycosidic bond in S-adenosylhomocysteine (SAH). Thus, Pfs converts SAH to adenine and S-ribosyl homocysteine. In a final step, S-ribosyl homocysteine undergoes cleavage to homocysteine and autoinducer-2. Homocysteine can re-enter this pathway after methylation to generate methionine, which MetK can convert to SAM.

[0287] Catabolism of SAH is a salvage pathway for recycling metabolic intermediates (adenine and homocysteine). Some bacteria, however, eliminate SAH by removing adenosine directly from SAH to generate homocysteine. Cells that use this second mechanism do not produce autoinducer-2. In the pathway shown in Figure 15, the enzyme responsible for conversion of S-ribosyl homocysteine to 4,5-dihydroxy-2-cyclopenten-1-one or 4-hydroxy-5-methyl-2h-furan-3-one was not previously identified, cloned, or purified.

[0288] LuxS is involved in the pathway shown in Figure 15, and SAM and SAH are involved in AI-2 production. The structure of AI-2 can be 4,5-dihydroxy-2-cyclopenten-1-one or 4-hydroxy-5-methyl-2h-furan-3-one, in which case LuxS is the uncharacterized enzyme that acts on S-ribosyl homocysteine. Second, LuxS could act on one of the intermediates to make AI-2. LuxS would represent a branch point off the known pathway.

[0289] To confirm that LuxS is involved in the conversion of SAM to AI-2, the gene encoding the *S. typhimurium* LuxS protein was cloned, overexpressed and the *S. typhimurium* LuxS protein was purified. This protein was used in combination with dialyzed cell-free extracts prepared from a *S. typhimurium luxS* null mutant to show that addition of SAM and LuxS protein could restore AI-2 production to dialyzed LuxS- cell extracts. Reaction mixtures were prepared containing 10 mM Sodium Phosphate buffer pH 7.0, dialyzed *S. typhimurium* LuxS- cell extract and SAM. Purified LuxS protein was added to some of these mixtures. The reactions were incubated at room temperature for 60 min, followed by centrifugation in a 5000 MWCO centricon. The material with MW < 5000 was added to the standard *V. harveyi* bioassay as previously described. Dialyzed LuxS- cell extracts to which SAM was added or extracts containing LuxS protein without the addition of SAM produced no AI-2 activity. However, identical extracts to which LuxS protein and SAM had been added produced AI-2 that resulted in over 500-fold stimulation in light production in the bioassay.

[0290] Further investigation showed that SAM is not the direct substrate for LuxS, and that LuxS must act at a step subsequent to the conversion of SAM to SAH (Figure 15). It was determined that AI-2 was not produced if SAM was added directly to LuxS protein, however activity was produced by pre-incubation of SAM with the LuxS- extracts, filtration, and subsequent addition of LuxS protein to the filtrate. Importantly, these studies indicate that SAM can react with an element in the cell extract before it can be used by LuxS to make AI-2. Presumably, the SAM dependent methyl transferases present in the cell extract use SAM as a methyl donor and convert it to SAH in the process. To verify this, SAH was substituted for SAM in an *in vitro* assay. Addition of SAH to the *in vitro* assay resulted in much greater AI-2 production than when SAM was added. This result indicates that LuxS functions in the pathway subsequent to the conversion of SAM to SAH. Again, addition of SAH directly to LuxS protein is not sufficient for production of AI-2 activity, while pre-incubation of SAH with dialyzed LuxS- extracts followed by filtration and subsequent addition of LuxS protein to the filtrates does result in AI-2 production. Presumably SAH is converted to S-ribosyl homocysteine and then LuxS acts to produce AI-2.

[0291] The proposed pathway shown in Figure 15 is not a salvage pathway for recycling secondary metabolites, but rather is the pathway for production of AI-2. According to the invention, AI-2 is a derivative of ribose. It is noteworthy that, in *V. harveyi*, LuxP, the primary sensor for AI-2, is a homologue of the *E. coli* and *S. typhimurium* ribose binding protein.

#### EXAMPLE 6 - Bioassay for Inhibitor of the AI-1 Mediated Quorum Sensing Pathway

[0292] The quantitative assay for detection of an autoinducer-1 quorum sensing system derives from a previous report by in M. Manefield et al, Microbiol 145, 283-291(1999).

[0293] In one example of this bioassay, test compounds such as the halogenated furanones of Structure VII are dissolved in ethanol for use in the current assay.

[0294] Synthetic OHHL (N-3-(oxohexanoyl)-L-homoserine), the AI-1 autoinducer in *V. fischeri*, prepared according to the method of A. Eberhard et al. Arch Microbiol 146, 35-40 (1986), is dissolved in ethyl acetate for use in the assay. [3H]OHHL is

prepared based on the method of H. Kaplan et al., J Label Compd Radiopharm 22, 387-395 (1985).

[0295] Bioluminescence is quantified as relative light units on a liquid scintillation counter. After addition of OHHL (10 nM to 100 nM OHHL) and the test compounds (up to 100  $\mu$ M) to dilute 10 mL cultures (OD<sub>600</sub> <0.0005) the luminescence response of 100  $\mu$ L samples is measured in triplicate. Appropriate concentrations of ethanol solvent are incorporated into control treatments. Growth is monitored by following the OD<sub>600</sub> of 10 ml cultures in appropriate media from lag-, through exponential- into stationary-phase growth.

#### EXAMPLE 7 - Bioassay for Inhibitors of the Peptide-Mediated Quorum Sensing Mechanism

[0296] The assay for detection of a peptide-mediated quorum sensing system is based on a previous report described in M. Otto et al., FEBS Lett. 450, 257-262. (1999). *S. epidermidis* Tü3298 (DSM) is the wild-type test strain and the host for the promoter test plasmid. *S. aureus* strains tested for  $\delta$ -toxin production are *S. aureus* Newman, 8325-4, SA113, ATCC 12600, RN4220, and ATCC 33591 of Otto et al. (1999). *S. aureus* RN6390 is a prototypic strain from which the following mutant strains are derived: *S. aureus* RN6911 “agr-“ an isogenic mutant carrying an *agr::getM* mutation, “sar-” carrying a *sar::Tn917LTV1* mutation, and “agr-/sar-” carrying both a *sar::Tn917LTV1* and an *agr::tetM* mutation of A. L. Cheung et al., J. Clin. Invest. 94, 1815-1822 (1994). The promoter test plasmid inserted into *S. epidermidis* Tü3298 contains the promoterless pUB112 cat gene of R. Brückner et al., EMBO J. 4 2950-2300 (1985) adjacent to a multiple cloning site and carries an erythromycin-resistance gene *ermB* from transposon Tn551. Plasmid pRB594P3 is constructed by insertion of a BamHI-digested PCR product of the *agr* P3 region of *S. epidermidis* ATCC 14990 into the BamHI site of the multiple cloning site.

[0297] *S. epidermidis* cells grown in TSB or BM (‘basic medium’: 1% tryptone (Difco), 0.5% yeast extract (Gibco BRL), 0.5% NaCl, 0.1% K<sub>2</sub>HPO<sub>4</sub>, 0.1% glucose) are disrupted in 20 mM Tris-HCl (pH 7.8) by glass beads as described in C. Sizemore et al., Mol. Gen Genet. 227, 377-384 (1991). Cell debris is removed by centrifugation (10 min, 5000xg). Membrane fractions are prepared by additional ultracentrifugation of the crude cell extract at

10500xg for 1h. Surface-associated proteins are isolated by boiling cells at 100°C for 5 min and centrifugation (10 min, 5000xg). Surface proteins are isolated by incubating cells with lysostaphin for 10 min at 37°C and centrifugation (10 min, 5000xg). Chromosomal staphylococcal DNA is prepared according to the method of J. Mamur, J. Mol. Biol. 3, 208-218 (1961). Proteins are separated by tricine-SDS-PAGE according to H. Schägger, H. Anal. Biochem. 166, 368-379 (1987) using Bio-Rad Protean IIxi chambers and a separation length of 16 cm.

[0298] Crude peptides are isolated on a Waters 600 Multi Solvent Delivery System equipped with a Lambda Max Model 481 as detector. A semi-preparative column (Nucleosil C18, 4x250mm; 5 m; Grom, Herrenberg, Germany) is eluted at a flow rate of 3.5 ml/min with a linear gradient (10-100% B in A in 45 min; solvent A: 0.1% trifluoroacetic acid (TFA) in water; solvent B: 0.1% TFA in acetonitrile). The detection wavelength is 214 nm. The concentration of purified peptides, redissolved in dimethylsulfoxide (DMSO), is determined using analytical HPLC on a Kontron HPLC System with Kroma System 2000 software. An analytical column (Spherisorb ODS2 2x100 mm; 5 µM; Grom, Herrenberg, Germany) is eluted at a flow rate of 250 µl/min with a linear gradient (0-100% B in A in 30 min; solvent A: 0.1 % TFA in water; solvent B: 0.1% TFA in acetonitrile). The detection wavelength is 214 nm. A known amount of the (unmodified) peptide DSVASYF is used as a reference. The amount of delta-toxin is quantified using the same system. A Pharmacia Resource PHE 1 ml column is eluted with 1.5 column volumes of a linear gradient (0-100% of B in A; A: 0.1% TFA in water; B: 0.1% TFA in acetonitrile). The S. epidermidis delta-toxin is eluted using the same conditions on a ÄKTA explorer 100 system (Amersham Pharmacia Biotech, Freiburg, Germany). The isolated delta-toxin is chemically analyzed by ESI-MS.

[0299] CAT activity is determined according to the method of W.V. Shaw, (1975) Methods Enzymol. 43, 737-755. The assay mixture contained 100 mM Tris-HCL (pH 7.8), 0.1 mM acetyl-coenzyme A and 0.4 mg 5,5'-dithiobis-2-nitrobenzoic acid (DTNB/ml). Assays are performed in 96 well microtiter plates using a SpectraMax 340 microtiter plate reader (Molecular Devices, Sunnyvale, CA USA) with SpectraMaxPro software. Cell extract (5µl) and 5 mM chloramphenicol in 100% ethanol (5µl) (or 5 µl 100% ethanol in controls)

are added to 90  $\mu$ l of the assay mixture. Cell extracts are diluted 1:10 or 1:100 with 20 mM Tris-HCL (pH 7.8) when necessary. Absorption at 412 nm is measured every 15 s for 20 min. The linear part of the resulting curve is used to determine the CAT activity (absorption coefficient  $\epsilon = 13600$  1/M for DTNB). For calculation of the specific activity, protein contents of the cell extracts are determined using the Bio-Rad DC protein assay for detergent-containing samples (Bio-Rad Laboratories GmbH, Munich, Germany).

[0300] SDS-polyacrylamide gels are blotted onto nitrocellulose membranes (Schleicher and Schuell BA 83) using the semi-dry blotting technique. Blots are blocked overnight with 5% skim milk. The first antibody is applied for 2 h at a concentration of 1:20,000 (anti- $\alpha$ -toxin) or 1:40,000 (anti-protein A). After washing, the blots are incubated with anti-IgG-coupled HRP from Amersham Pharmacia (1:5000) for 1 h. All dilutions are made in Tris-buffered saline (TBS: 10mM Tris-HCl, pH 7.4, 150 mM NaCl). Signals are detected with the ECL detection system (Amersham Pharmacia Biotech, Freiburg, Germany).

[0301] Blood plates are prepared with sheep blood agar base (Oxoid) to which 5% defibrinated sheep blood is added. Samples are spotted onto filters, which are dried and then laid on agar plates and incubated at 37°C for at least 24 h.

#### EXAMPLE 8 - Antibiotic Susceptibility Assay

[0302] Antimicrobial susceptibility testing was conducted according to the National Committee for Clinical Laboratory Standards (NCCLS) reference method "Broth Dilution Procedure (Microdilution)" published January 1977 (UPDATE VERSION). The method yields a minimum inhibitory dose (MIC) required for complete inhibition of bacterial growth. MICs were determined using a dilution series of antibiotic, in which all of the wells contained a constant concentration of the inhibitor compound was maintained. In addition, before the instant susceptibility tests were performed, the inhibitor compounds were tested in the appropriate inhibitor screens (See, for example, Figure 32 for the AI-2 inhibitor 2-ethyl-4-hydroxy-5-methyl-3(2H)-furanone). Once their inhibitory activity is demonstrated in screens, the inhibitors were also tested for antibiotic activity. Experiments were conducted in 96 well microtiter plates using a final volume of 0.1 ml/well. Assays were conducted using either Mueller-Hinton broth, Todd-Hewitt broth, or Nutrient Broth (Difco). The bacterial



innoculum was prepared from a log phase overnight culture of *Streptococcus pyogenes* ATCC 19615 or *Staphylococcus aureus* ATCC 25923. Turbidity of the cultures was adjusted with saline to 0.05 McFarland standard ( $10^7$  CFU/ml), and 5 microliters of this inoculum was added to each well. The inhibitor compounds were prepared by dissolving a stock solution in water. Working stocks of quorum inhibitors compounds were made by diluting the stock solutions with bacterial broth. Sodium salts of the antibiotics were used in these experiments. A stock solution (10 mg/ml) was prepared as stipulated by the manufacturer by dissolving a weighed amount of antibiotic in water or DMSO. All antibiotics were added in two-fold serial dilutions to a series of wells to yield final concentrations ranging from 100 µg/ml to 0.1 µg/ml. The single exception was sulfisoxazole where the range of final doses ranged from 300 µg/ml to 2.5 µg/ml. Bacterial broth was used as the diluent. The range of antibiotic concentrations used were chosen to be at and below the MIC for the antibiotic alone. Cultures were sealed and incubated for 20 hours at 37°C. MIC values were determined by spectrophotometric measurements of absorbance at 600 nm. The MIC is defined as the lowest concentration of antibiotic that completely inhibits growth of 99% the test organism in the tubes. Fold-changes in MIC concentration were determined by comparing the MIC for antibiotic and quorum inhibitor compounds with the MIC measured using antibiotic alone.

[0303] Using the above antimicrobial assay, the combination of the quorum inhibitor compound 2-ethyl-4-hydroxy-5-methyl-3(2H)-furanone in combination with either vancomycin, ciprofloxacin or sulfisoxazole showed marked synergies against the test strain. The quorum inhibitor compound did not inhibit cell growth (Fig 33). Vancomycin alone had an Minimum Inhibitory Concentration (MIC) of approximately 100 µg/ml; when it was combined with the inhibitor compound at a concentration of 5 µg/ml it had approximately the same MIC, when it was combined with the quorum inhibitor compound at 12.5 µg/ml it had an MIC of approximately 1.6 micrograms/milliliter. Vancomycin had the same MIC when the concentration of the quorum inhibitor compound was increased to 50 µg/ml. Finally, with the inhibitor alone, the MIC was greater than 100 µg/ml. Against the same test strain, ciprofloxacin alone has a MIC of 0.8 µg/ml. In combination with the same quorum inhibitor molecule at a concentration of 25 µg/ml, ciprofloxacin displayed a MIC of 0.4 µg/ml. Again, when the inhibitor was tested alone the MIC was greater than 100 µg/ml. Against the same

test strain sulfisoxazole alone exhibited an MIC of approximately 300 micrograms per milliliter, while in the presence of a concentration of 100 micrograms per milliliter of the quorum inhibitor compound it exhibited a MIC of approximately 100 µg/ml. As before, the inhibitor compound alone demonstrated and MIC of greater than 100 micrograms per milliliter. Against the other test strain, *Staphylococcus aureus*, ampicillin alone had a MIC of 1.6 µg/ml, while in the presence of the inhibitor compound (25 µg/ml) the antibiotic had a MIC of 0.4 µg/ml.

#### EXAMPLE 9 - Isolation of Halogenated Furanones

[0304] Many of the halogenated furanones of Structure VII are extracted from their host bacteria according to protocols established by R. de Nys, Tetrahedron 49, 11213-11220(1993). Algal tissue is frozen, freeze-dried, extracted with dichloromethane and reduced in vacuo. Pure compounds are isolated from this crude extract by vacuum liquid chromatography and HPLC, and identified by <sup>1</sup>H and <sup>13</sup>C NMR spectroscopy.

#### EXAMPLE 10 - Preparation of Inhibitors of the Peptide Quorum-Sensing Mechanism

[0305] Cyclic peptides of the formula (cyclo)-YSTCDFIM; (cyclo)-GVNACSSLF; (cyclo)-GVNASSSLF; and (cyclo)-GVNA(DAPA)SSLF, in which the C-terminal carbonyl group forms a thiolactone with the sulfur atom of the cysteine residue (YSTCDFIM and GVNACSSLF); a lactone group with the oxygen atom of the first serine residue (GVNASSSLF); or an amide bond with amino group of the diaminopropionic acid (DAPA) residue (GVNA(DAPA)SSLF; are synthesized through use of the Fmoc/tBu strategy on Tritylresin (PepChem: Clausen and Goldammer, Tübingen, Germany). The sequence of the peptide is DSVXASYF, with cysteine (C), serine (S) or 1,3-diaminopropionic acid (Dpr) in the X position. The corresponding protected amino acids for the synthesis of cyclic peptides are Fmoc-Cys(Mmt)-OH, Fmoc-Ser(Trt)-OH (both cleavable with TFA:TIS in dichloromethane) and Fmoc-Dpr(Dde)-OH (cleavable with hydrazine). The cyclic peptides are synthesized and purified according to M Otto, et al. (1998) FEBS Lett. 424, 89-94. The purity of peptides (> 90%) is controlled by RP-C18 chromatography and ESI-MS.

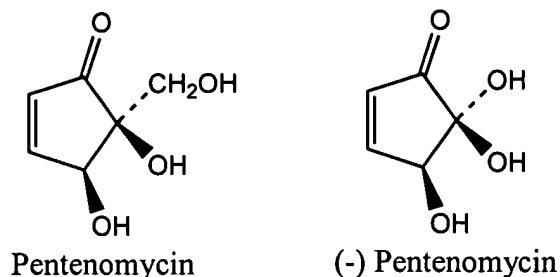
#### EXAMPLE 11 - Characterization and Biosynthesis of an AI-2 Analog

[0306] The invention further provides a method for an *in vitro* procedure for large-scale production of pure AI-2. As indicated in Figure 15, SAH is a precursor in the LuxS dependent biosynthesis of AI-2. Furthermore, LuxS does not act directly on SAH. Prior to the action of LuxS, some enzyme in dialyzed cell extracts must first act on SAH to convert it to S-ribosyl homocysteine by Pfs. Therefore the substrate for LuxS is S-ribosyl homocysteine.

[0307] To confirm that LuxS acts on S-ribosyl homocysteine, the Pfs enzyme can be purified and used to convert SAH to S-ribosyl homocysteine. Toward this end, the *pfs* gene has been cloned from *S. typhimurium* 14028 placed into the overexpression vector pLM-1. The Pfs enzyme will be overexpressed and SAH will be added to purified Pfs to produce S-ribosyl homocysteine. The conversion of SAH to S-ribosyl homocysteine will be confirmed by reverse phase HPLC analysis (SAH is UV active while S-ribosyl homocysteine is not). Subsequently, the S-ribosyl homocysteine produced by Pfs will be added to purified LuxS. Following incubation, the mixture will be filtered over a 5000 MWCO centricron. The filtrate will be tested for AI-2 activity in the previously described *V. harveyi* bioassay.

#### Regulation of LuxP or LuxQ Activity

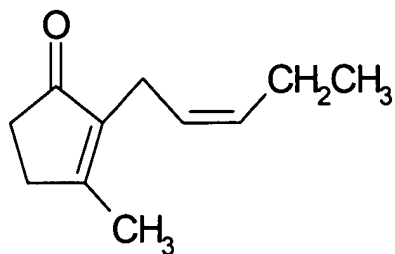
[0308] It is an object of the invention to provide compounds and methods for regulating the activity of autoinducer-2 receptors such as LuxP or LuxQ. Compounds of the invention include those that interact with LuxP or LuxQ. Specifically, the invention provides a method for the regulation of LuxP or LuxQ protein resulting in the regulation of bacterial cell growth or the regulation of bacterial pathogenicity by regulating the expression of a factor associated with bacterial virulence. The method of the invention envisions contacting LuxP or LuxQ with pentenomycin, or derivatives thereof, such that LuxP or LuxQ activity is regulated. Pentenomycin has previously been identified as having antibiotic activity. The present study provides the first data indicating that pentenomycin acts as an inhibitor of AI-2 initiated activation of a biochemical pathway. The chemical structure of pentenomycin, and derivatives thereof (see below), is/are similar to that of the structure of autoinducer-2 as identified in the present study.



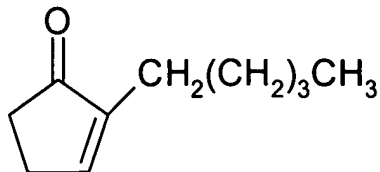
[0309] The present invention provides a basis for the rational design of derivatives and analogs of AI-2 for regulating bacterial growth and pathogenicity. Such analogs and derivatives can be used to regulate the activity of those proteins involved in the autoinducer-2 signaling pathway, such as LuxP or LuxQ.

[0310] An autoinducer-2 (AI-2) molecule of the invention can interact with LuxP, the protein encoded by the homologue of the *luxP* gene of pathogenic bacteria such as *V. cholerae*, *S. typhimurium* and *E. coli*. In turn, the AI-2-LuxP complex can interact with LuxQ, which is the protein product encoded by the *luxQ* gene. The AI-2-LuxP-LuxQ interaction can promote luminescence in bacteria such as *Vibrio spp* by activating the expression of genes involved in luminescence. In addition, the AI-2-LuxP-LuxQ interaction has been linked to the activation of biochemical pathways required for bacterial pathogenicity. Thus, the invention provides a method for controlling bacterial gene expression and for regulating bacterial pathogenicity by regulating AI-2-LuxP-LuxQ interactions using an AI-2 analogue, such as pentenomycin. For example, an AI-2 analogue can compete with endogenous AI-2 for binding to LuxP or LuxQ proteins thereby providing a means for regulating the activity of the protein.

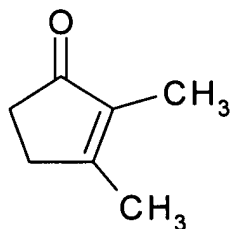
[0311] The present invention further provides examples of AI-2 analogs capable of inhibiting AI-2 activated luminescence in the *V. harveyi* luminescence assay (see Figures 16-18). These AI-2 analogs have not previously been shown to have antimicrobial activity or act as an inhibitor of AI-2 initiated activation of a biochemical pathway. Examples of such analogs include:



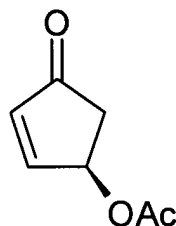
Cis-Jasmone



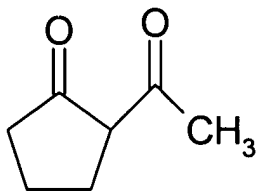
2-pentyl-2-cyclopenten-1-one



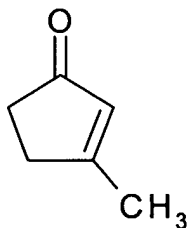
2,3-dimethyl-2-cyclopenten-1-one



4S-Acetoxy-2-cyclopenten-1-one



2-acetylcyclopentanone



3-methyl-2-cyclopenten-1-one

[0312] Figure 16 shows the results of screening assays of AI-2 analogues. Figure 16 panels A and B show the results of a 6 hour assay using compounds 6, 9, 15 and 18 (panel A) and compounds 3, 4, 7 and 13 (panel B) on test strain BB170. Figure 16 panels C and D show the effect of the same compounds on control strain JAF78. Strain BB170 synthesizes AI-2 starting at 4.5 hours into the assay. Light produced at the start of the assays is from AI-2

carried over from cells diluted from stationary phase. Strain JAF78 is a mutant strain in which light production is “locked on” and is unaffected by the presence or absence of autoinducer. Increase in light over the time course reflects increase in cell population density. Compounds 6,9,15 and 18 produced approximately a 100-fold decrease in light emission from the no-compound control at the 6 hour time point in BB170 but no significant change in light emission in JAF78 (panels A&C). Direct counts of cells plated from each treatment showed no significant differences in cell viability. Compounds 3,4,7 and 13 produced no significant difference in light emission in either strain (panels B&D).

**[0313] LuxO Regulation of Expression.** At low cell density, in the absence of autoinducer-2, the LuxQ sensor acts as a kinase. The sensor autophosphorylates conserved His residues and transfers the phosphoryl group to conserved Asp residues in attached response regulator domains. Thus, the first phosphotransfer event is intra-molecular. Subsequently, inter-molecular phospho-transfer occurs from the LuxQ sensor to a conserved His residue of the phosphorelay protein LuxU. In the final step, the phosphoryl group is transferred to the conserved Asp in the response regulator protein LuxO. Phosphorylation of LuxO activates the protein, and its function is to cause repression of the *luxCDABEGH* operon. Therefore, at low cell density, the bacteria make no light. At high cell density, in the presence of autoinducer-2, LuxQ activity is altered, switching from kinase to phosphatase activity. In this mode, the sensor drains phosphate out of the system. The phosphatase activity of the sensor results in rapid elimination of LuxO-phosphate, and the dephosphorylated form of LuxO is inactive. Therefore, at high cell density, no repression of *luxCDABEGH* occurs, and the bacteria emit light.

**[0314]** *V. harveyi* uses this complex quorum sensing system, specifically the LuxPQ/AI-2 quorum sensing circuit, to communicate between species. Therefore, *V. harveyi* monitors not only its own cell-population density but also that of other bacteria. This ability allows *V. harveyi* to regulate behavior based on whether it exists alone or in consortium. *luxS*, the gene encoding the AI-2 synthase, belongs to a highly conserved family of genes that specify AI-2 production in a wide range of both Gram-negative and Gram-positive bacteria, including *E. coli*, *S. typhimurium*, *Salmonella typhi*, *Vibrio cholerae*, *Yersinia pestis*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Enterococcus faecalis*, and *Bacillus subtilis*.

Thus, various species of bacteria can use AI-2 for inter-species communication. Moreover, the present study indicates that the presence of AI-2 initiates a cascade of events culminating in the derepression (*i.e.*, activation) of various genes. It is an object of the invention to provide compounds and methods for regulating AI-2 activity such that the derepression/activation of downstream genetic elements is also regulated. To that end, the present study indicates that LuxO activates transcription of downstream target genes including the production of siderophore, as well as colony morphology (Table 4 and Figure 20). These are the first examples of quorum sensing regulated phenotypes, other than Lux, in *V. harveyi* clearly indicating that AI-2 quorum sensing controls potentially pathogenic phenotypes .

[0315] In the present study, mutations in *luxO* (the gene encoding the LuxO protein) and/or *rpoN* (the gene encoding the  $\sigma^{54}$  protein) were tested to determine if they affected siderophore production in *V. harveyi*. The Schwyn and Neilands chromazurol S assay was used to measure siderophore released by different *V. harveyi* strains. The S assay quantitatively measures siderophore by optically assessing the color change that chromazurol S undergoes when it loses its chelated ferric ion to siderophore present in spent culture fluids. Regulation of siderophore production in many species of bacteria including *E. coli* and *V. cholerae* is under the control of the ferric uptake regulation (Fur) protein. In these cases, under iron-rich conditions, the Fur protein binds Fe(II) ions and represses the transcription of genes required for siderophore biosynthesis and transport. De-repression of these genes occurs during periods of iron deprivation, when Fur is not bound to Fe(II) The results presented in Table 4 indicate that LuxO and  $\sigma^{54}$  have a role in activating the production of siderophores.

Table 4

LuxO and  $\sigma^{54}$  regulate siderophore production in *V. harveyi*

V. harveyi strain	Genotype	Plac-rpoN <sup>a</sup>	Siderophore units <sup>b</sup>
BB120	wild type	-	8 ± 3
JAF78	<i>luxO</i> :: Cm <sup>r</sup>	-	7 ± 4
JAF548	<i>luxO</i> D47E	-	50 ± 5
BNL240	<i>rpoN</i> ::Cm <sup>r</sup>	-	3 ± 3
BNL240	<i>rpoN</i> ::Cm <sup>r</sup>	+	6 ± 3
BNL244	<i>luxO</i> D47E, <i>rpoN</i> ::Cm <sup>r</sup>	-	4 ± 1
BNL244	<i>luxO</i> D47E, <i>rpoN</i> ::Cm <sup>r</sup>	+	25 ± 3

<sup>a</sup>The wild type *V. harveyi* *rpoN* gene was expressed under control of the *lac* promoter from plasmid pBNL2090 (Table 4).

<sup>b</sup>Siderophore production was measured using the chromazurol S assay (Schwyn and Neilands, 1987). Siderophore units were calculated according to the method of Payne (1994), and normalized for cell number using the formula: 100 X [(OD<sub>630</sub> (media control) – OD<sub>630</sub> (spent culture fluid)) / OD<sub>600</sub> (cell culture)]. Values shown are the mean ± SEM of three independent experiments.

[0316] Wild type strain BB120, the *-luxO* strain JAF78, and the *rpoN*::Cm<sup>r</sup> null strain BNL240 all produce similar amounts of siderophore (3 to 8 units) when grown in AB minimal medium. In contrast, the presence of activated *luxO* D47E in JAF548 increases siderophore production to 50 units. This result indicates that phospho-LuxO activates siderophore production. Disruption of *rpoN* in the *luxO* D47E background (strain BNL244) reduces siderophore production to wild type levels (4 units), indicating that similar to what was shown above for Lux regulation, phospho-LuxO controls siderophore production when wild type  $\sigma^{54}$  is present. *In trans* introduction of wild type *rpoN* into the *luxO* D47E, *rpoN*::Cm<sup>r</sup> strain complements the defect. In this case, siderophore production increased to 25 units, approaching that of the *luxO* D47E strain. These results demonstrate that the activated form of LuxO, along with  $\sigma^{54}$ , has a role in regulation of siderophore production.

[0317] In addition to the siderophore production phenotype, the present study shows that *V. harveyi* mutants possessing a constitutively activated LuxO (*i.e.*, LuxO D47E



or LuxN L166R) also consistently exhibit an altered colony morphology that is similar to the rugose colony morphology described for *V. cholerae* and the opaque colony morphology described for *Vibrio parahaemolyticus*. In *V. cholerae*, the rugose phenotype requires a large gene cluster called *vps* that is necessary for the production of the exopolysaccharide rugose polysaccharide. In *V. parahaemolyticus* a homologue of the *V. harveyi* LuxR transcriptional activator protein called OpaR is involved in the switch to the opaque phenotype. *Vibrio cholerae* is the causal organism of the diarrheal disease cholera and the rugose variants of *V. cholerae* have been shown to produce an exopolysaccharide matrix. The rugose polysaccharide has been shown to confer increased resistance to a variety of agents such as chlorine, bioacids, and oxidative and osmotic stresses. In addition, the rugose phenotype promotes biofilm formation thereby increasing the survival of the organism in aquatic environments.

[0318] Figure 20 is a photograph showing the colony morphologies of various *V. harveyi* strains. Colonies of wild type *V. harveyi* and the *rpoN*::Cm<sup>r</sup> null strain are smooth and glassy in appearance, while colonies of the *luxO* D47E strain are wrinkled and opaque. The figure shows that the colony morphology phenotype caused by the activated *luxO* D47E protein is dependent upon the presence of wild type *rpoN* because strain BNL244 (*luxO* D47E, *rpoN*::Cm<sup>r</sup>) has the wild type smooth colony morphology. Similar to that observed for rugose strains of *V. cholerae*, the *V. harveyi luxO* D47E mutant forms a pellicle when grown in liquid culture. Pellicle formation is also dependent on wild type *rpoN*. Identical results were obtained when the “locked” LuxN L166R strain JAF549 was used in place of the LuxO D47E strain JAF548. The fact that a single amino acid change in LuxO or LuxN can affect various phenotypes including siderophore production and colony morphology indicates that LuxO and  $\sigma^{54}$  are involved in the regulation of multiple target genes.

[0319] The inhibition of an AI-2 mediated response was further measured in the *V. harveyi* bioassay. Compound QXP031 was tested against *V. harveyi* tester and control strains. As shown in Figure 21, at the concentrations indicated, compound QXP031 specifically inhibited AI-2 mediated luciferase expression in *V. harveyi* as monitored by bioluminescence intensity. The results are normalized to cell count and any minimal effects seen in the control strain. The fold difference in bioluminescence intensity at the different

drug concentrations versus no drug control is indicated above the appropriate bars in Figure 21.

[0320] In addition, the effect of a compound of the invention on the expression of a virulence factor was measured in a CAMP assay. Many streptococci produce a diffusible extracellular protein (CAMP) that acts synergistically with staphylococcal beta lysin to lyse erythrocytes on trypticase soy agar plates containing sheep blood. Hemolysis of erythrocytes is an important component of the virulence response in many pathogenic bacteria. A single straight streak of a beta lysin producing *S. aureus* strain is made. Streptococci to be tested are streaked perpendicular to the *S. aureus* streak. After overnight incubation a positive test is characterized by an arrowhead-shaped zone of complete hemolysis in the area into which both staphylococcal beta lysin and streptococcal CAMP factor have diffused. Compound QXP031 was tested in a CAMP assay employing *S. aureus* and *S. pyogenes* pathogenic bacteria. Application of QXP031 resulted in a negative CAMP score as shown in Figure 22.

[0321] Proteinase activity is an important component of the virulence response in many pathogenic bacteria. Skim milk containing agar plates can be used to score for proteinase activity in a variety of bacteria. Proteolytic activity is scored as a clearing zone surrounding a test inoculum. The clearing zone is the result of the proteolytic breakdown of the milk proteins suspended in the agar plates. Regions where milk protein is not broken retain a milky opaque appearance. Figure 23 shows the inhibition of *S. pyogenes* proteolytic activity by QXP031.

[0322] Thus, the present invention to provide compounds and methods for regulating the effect of AI-2 on such targets. Provided herein are compounds of structure I, II, III or IV and pharmaceutical compositions comprising such compounds and methods of using the compounds and compositions of the invention to regulate bacterial growth and virulence by regulating the activity of AI-2 and proteins that interact with AI-2. Thus, the invention provides a mechanism for the control of bacterial growth, such as by inhibition of bacterial growth, utilizing the compounds of the invention. The invention further provides a mechanism to not only control bacterial growth but also to control those pathways involved in expression of phenotypes associated with bacterial virulence and pathogenicity such as siderophore production and rugose polysaccharide production.

#### EXAMPLE 12 - Biofilm inhibition

[0323] Biofilm formation was measured using a microtiter-based assay. Free-swimming (planktonic) bacteria were cultured in AB or LB media supplemented with glucose in 96 well microtiter dishes. Biofilms typically formed along the walls of the wells at the interface of the liquid and air phase. At the end of the incubation, aliquots of a crystal violet solution (stock 0.1%) were added to the wells to stain both the adherent and the non-adherent cells. The wells were then washed with deionized water to remove non-adherent cells, and the bound crystal violet solubilized with 200 microliters of water. Absorbance reading at 600nm (A600) were used to quantitate the amount of crystal violet dye that had adhered to the biofilms. When indicated, synthetic AI-2 was added to the wells at the beginning of the cultures.

[0324] Various genotypes of *V. harveyi* were tested for biofilm formation in the absence of any exogenous AI-2 (Figure 26). The MM32 strain (LuxN-, LuxS-) formed more biofilm than either the wild-type *V. harveyi* (strain BB120) or *V. harveyi* indicator strain BB170 which contains an inactivating mutation in LuxN. The results were the same regardless of whether AB media with glucose (Panel A) or LB media supplemented with glucose (Panel B) was used. These observations suggest that more biofilm is produced by *V. harveyi* when the endogenous synthesis of AI-2 is eliminated.

[0325] As shown in Figure 26, strains of *V. harveyi* were used to test the effects of exogenous 4-hydroxy-5-methyl-2h-furan-3-one on biofilm formation. Various amounts of synthetic AI-2 were added to the initial free-swimming bacterial cultures in LB media supplemented with glucose, and biofilm formation measured at the end of the incubation. The results indicate that AI-2 affects biofilm formation in all *V. harveyi* strains, and that the results are reproducible for each genotype. In the MM32 strain, low doses of AI-2 block biofilm formation. In contrast, addition of the same low doses of AI-2 to wild-type *V. harveyi* (strain BB120) or *V. harveyi* indicator strain BB170 increases the extent of biofilm that forms. Higher concentrations of AI-2 stimulated biofilm formation in all *V. harveyi* strains tested.

[0326] The effects of AI-2 on biofilm formation were also evaluated in *Pseudomonas aeruginosa* (ATCC 27853) (Figure 27). Biofilm formation by this organism is of particular interest because of the association of *Pseudomonas aeruginosa* biofilms with decreased lung capacity in subjects with cystic fibrosis. Using AB media with glucose as the media, addition of exogenous synthetic AI-2 caused a dose-dependent increase in the level of biofilm formation by *P. aeruginosa*. The magnitude and dose-response to AI-2 was found to be pH-dependent in this system.

#### Materials and Methods

[0327] **Strains and Media.** The bacterial strains used and their genotypes and phenotypes are listed in Table 5.

Table 5  
Bacterial Strains; Their Genotypes and Relevant Phenotypes

Strain	Genotype	Relevant phenotype
<i>S. typhimurium</i> LT2		Wild type
<i>E. coli</i> O157		Wild type
<i>E. coli</i> MG1655	F-, ilvG, rfb-50	Wild type
<i>E. coli</i> MC4100	(lac)U169, araD139, rpsL, thi	LacZ-
<i>E. coli</i> DH5 $\alpha$	supE44, hsdR17, recA1, endA1, gyrA96, thi-1, relA1	AI-2
<i>V. harvey</i> BB170	<i>luxN</i> ::Tn5	Sensor 1-, Sensor 2+
<i>V. harveyi</i> BB152	<i>luxL</i> ::Tn5	AI-1-, AI-2+
<i>V. harveyi</i> JAF305	<i>luxN</i> ::Cm5	Sensor 1-, Sensor 2+

[0328] Luria broth (LB) contained 10 g Bacto Tryptone (Difco), 5 g Yeast Extract (Difco) and 10 g NaCl per liter (Sambrook *et al.*, 1989). The recipe for Autoinducer Bioassay (AB) medium has been reported previously (Greenberg *et al.*, Arch. Microbiol. 120: 87-91, 1979). LM medium (L-Marine) contains 20 g NaCl, 10 g Bacto Tryptone, 5 g Bacto Yeast

Extract and 15 g Agar per liter (Bassler *et al.*, 1994, *supra*). Regulation of AI-2 production similar to that reported here was also observed with the ATCC strain *Salmonella enterica* Serovar Typhimurium 14028, an independent clinical isolate of *Salmonella enterica* Serovar Typhimurium, and nine other *Salmonella enterica* serovars (other than Typhimurium).

**[0329] Growth Conditions for *S. typhimurium* LT2 and Preparation of Cell-free Culture Fluids.** *S. typhimurium* LT2 was grown overnight in LB broth with shaking at 30°C. The next day, 30 µl of the overnight culture was used to inoculate 3 ml of fresh LB broth. In cultures containing additional carbon sources, at the time of inoculation, 20% sterile stock solutions were added to give the specified final concentrations. Following subculturing of the cells, the tubes were shaken at 200 rpm at 30°C for the time periods indicated in the text. Cell-free culture fluids were prepared by removal of the cells from the culture medium by centrifugation for 5 min at 15,000 rpm in a microcentrifuge. The cleared supernatants were passed through 0.2 µm cellulose acetate Spin X filters (CoStar, Cambridge, MA) by centrifugation for 1 min at 8000 X g. Samples were stored at -20°C. Similar results to those reported here were obtained when we grew the *S. typhimurium* at 37°C. The preparation of cell-free culture fluids from *V. harveyi* strains has already been reported (Bassler *et al.*, 1993, *supra*; Bassler *et al.*, 1997, *supra*).

**[0330] Density-dependent Bioluminescence Assay.** The *V. harveyi* reporter strain BB170 (Sensor 1<sup>-</sup>, Sensor 2<sup>+</sup>) (Bassler *et al.*, 1993, *supra*) was grown for 12 h at 30°C in AB medium, and diluted 1:5000 into fresh AB medium. Luminescence was measured as a function of cell density by quantitating light production at different times during growth with a Wallac Model 1409 liquid scintillation counter (Wallac Inc., Gaithersburg, MD). The cell density was measured by diluting the same aliquots of cells used for measuring luminescence, spreading the dilutions onto solid LM medium, incubating the plates overnight at 30°C, and counting the resulting colonies the following day. Relative Light Units are (counts min<sup>-1</sup> ml<sup>-1</sup> x 10<sup>3</sup>)/(colony forming units ml<sup>-1</sup>). Cell-free culture supernatants from *V. harveyi* or *S. typhimurium* strains were added to a final concentration of 10% (v/v) at the time of the first measurement. In control experiments, 10% (v/v) of AB medium, LB medium or LB medium containing 0.5% glucose was added instead of cell-free culture fluids.

[0331] ***S. typhimurium* Autoinducer Activity Assay.** The quorum-sensing signaling activity released by *S. typhimurium* LT2 was assayed following growth under various conditions. 10 µl of cell-free culture fluids from *S. typhimurium* LT2 grown and harvested as described above were added to 96-well microtiter dishes. The *V. harveyi* reporter strain BB170 was grown overnight and diluted as described above. Ninety microliters of the diluted *V. harveyi* cells were added to the wells containing the *S. typhimurium* cell-free culture fluids. Positive control wells contained 10 µl of cell-free culture fluid from *V. harveyi* BB152 (AI-1<sup>-</sup>, AI-2<sup>+</sup>) (Bassler *et al.*, 1993, *supra*). The microtiter dishes were shaken in a rotary shaker at 200 rpm at 30°C. Light production was measured hourly using a Wallac Model 1450 Microbeta Plus liquid scintillation counter designed for microtiter dishes (Wallac Inc., Gaithersburg, MD). In these experiments, the cell density was not measured at each time point. Rather, to ensure that increased light production was due to a signaling activity and not a growth medium component, the luminescence production by *V. harveyi* in wells containing cell-free culture fluids was compared to that produced by *V. harveyi* in wells containing 10 µl of the identical growth medium alone. Data are reported as fold-stimulation over that obtained for growth medium alone.

[0332] **Factors Controlling Signal Production in *S. typhimurium*.** *S. typhimurium* LT2 was grown for 6 h in LB containing 0.5% glucose as described above. The mid-exponential phase culture was divided into several identical aliquots. One aliquot of cells was grown to stationary phase (24 h at 30°C with shaking). In the remaining aliquots, the cells were removed from the LB-glucose growth medium by centrifugation for 5 min at 15,000 rpm in a microcentrifuge. The resulting cell pellets were resuspended at an OD<sub>600</sub> of 2.0 in either LB, LB + 0.5% glucose, LB at pH 5.0, or in 0.1M NaCl, or 0.4M NaCl (in water). The resuspended cells were shaken at 30°C or 43°C for 2 h. Cell-free fluids were prepared from the stationary phase culture, and from the cells that had been resuspended and incubated in the various media or the osmotic shock solutions. The cell-free fluids were tested for signaling activity in the *S. typhimurium* activity assay as described above.

[0333] **Effects of Growth Phase, pH, Glucose Concentration and Osmolarity on Autoinducer Production by *S. typhimurium*.** *S. typhimurium* LT2 was grown at 30°C for various times in LB containing limiting (0.1%) and non-limiting (1.0%) glucose

concentrations. At the times specified in the text, the cell number was determined by plating dilutions of the *S. typhimurium* cultures onto LB medium and counting colonies the following day. The pH of the two cultures was measured, and the percent glucose remaining in each culture was determined using the Trinder assay as described in Example 1. Cell-free culture fluids were prepared from the LB-glucose cultures as described above. The same cells from which the cell-free culture fluids were prepared were resuspended in 0.4M NaCl osmotic shock solution and shaken at 200 rpm, 30°C for 2 h. We determined that this timing was optimal for production of autoinducer. The cells were removed from the osmotic shock solution by centrifugation at 15,000 rpm for 5 min in a microcentrifuge. Cell-free osmotic shock fluids were prepared from the resuspended cells exactly as described for cell-free culture fluids. Signaling activity in both the cell-free culture fluids and the cell-free osmotic shock fluids was assayed as described above. In experiments in which the pH was maintained at 7.2, the cells were grown in LB + 0.5% glucose containing 50 mM MOPS at pH 7.2. The pH was adjusted every 15-30 min using 1 M MOPS pH 7.2. In experiments performed at pH 5.0, LB broth was maintained between pH 5.0 and 5.2 with 1M NaOH.

**[0334] Requirement for Protein Synthesis in Signal Production, Release and Degradation by *S. typhimurium* LT2.** *S. typhimurium* LT2 was pre-grown in LB containing 0.5% glucose at 30°C to an OD<sub>600</sub> of 2.5 (approximately 6-8 h). The culture was divided into four identical aliquots. Two aliquots were treated with 100 µg/ml Cm for 5 min at room temperature after which the cells were harvested by centrifugation at 15,000 rpm for 5 min. One Cm-treated cell pellet was resuspended in 0.1M NaCl containing 30 µg/ml Cm, and the second pellet was resuspended in 0.4M NaCl containing 30 g/ml Cm. Each of these pellets was resuspended to a final OD<sub>600</sub> of 2.0. The remaining two culture aliquots were not treated with Cm. Instead, the cells in these two aliquots were harvested by centrifugation and resuspended in 0.1M and 0.4M NaCl exactly as described for the Cm-treated cells. The cell suspensions were incubated at 30°C with shaking. At the times indicated in the text, 1.5 ml aliquots were removed from the cell suspensions and cell-free osmotic shock fluids were prepared by the procedure described above.

**[0335] Analysis of the Effect of Autoinducer on SdiA Regulated Gene Expression.** A sequence that includes the *ftsQ1p* and *ftsQ2p* promoters (Wang *et al.*, 1991,

*supra*) was amplified from *E. coli* MG1655 chromosomal DNA using the following primers: *ftsQ1p*, 5'-CGGAGATCTGCGCTTTCAATGGATAAACTACG-3'; *ftsQ2p*, 5'-CGCGGATCCTCTTCTTCGCTGTTTCGCGTG-3'. The amplified product contained both the *ftsQ* promoters and the first 14 codons of the *ftsQ* gene flanked by *Bam*HI and *Bgl*II sites. The *ftsQ1p2p* PCR product was cloned into the *Bam*HI site of vector pMLB1034 (Silhavy *et al.*, Experiments with Gene Fusions, Cold Spring Harbor Press, 1984) to generate a *lacZ* fusion that contained the promoters, ribosome-binding site, and initiation codon of *ftsQ*. A correctly oriented clone, pMS207, and a clone containing the *ftsQ1p2p* insert in the opposite orientation, pMS209, were chosen for further analysis. Both inserts were sequenced to ensure that no errors were introduced during the PCR reaction.

[0336] For *ftsQ* regulation in *E. coli*, the plasmids pMS207 and pMS209 were transformed into *E. coli* strain MC4100 (Silhavy *et al.*, 1984, *supra*), and the transformants were grown overnight in LB containing 100 mg/L ampicillin at 30°C with aeration. For *rck* regulation, *S. typhimurium* strains BA1105 (*rck::MudJ*) and BA1305 (*rck::MudJ sdiA*) were grown overnight in LB containing 100 mg/L kanamycin at 30°C with aeration. The overnight cultures were diluted 20-fold into fresh medium and grown for an additional 4.5 h. At this time, each culture was divided into five identical aliquots and 10% (v/v) of one of the following was added to each aliquot: LB, 0.4M NaCl, 0.4M osmotic shock fluids from *S. typhimurium* LT2, *E. coli* O157 or *E. coli* strain DH5α (negative control). The osmotic shock fluids were prepared as described above, following pre-growth of the *S. typhimurium* LT2 and *E. coli* in LB containing 0.5% glucose for 6h. The cell suspensions were incubated at 30°C for 2 h, after which standard-galactosidase reactions were performed on the samples (Miller, A Short Course in Bacterial Genetics, Cold Spring Harbor Laboratory Press, 1992).

[0337] **Preparation of Cell-free Culture Fluids.** *E. coli* strains AB1157 and DH5α and *S. typhimurium* strain LT2 were grown at 30°C overnight with aeration in LB broth containing glucose at the concentrations specified in the text. The following morning, fresh LB medium containing the same concentration of glucose used for the overnight growth was inoculated at a 1:100 dilution with the overnight grown cultures. The fresh cultures were grown for various times at 30°C with aeration. Cell-free culture fluids were prepared by removing the cells from the growth medium by centrifugation at 15,000 rpm for 5 min in a



microcentrifuge. The cleared culture fluids were passed through 0.2 µm HT Tuffryn filters (Gelman) and stored at -20°C. Cell-free culture fluids containing *V. harveyi* Autoinducer-2 were prepared from *V. harveyi* strain BB152 (Autoinducer 1<sup>-</sup>, Autoinducer 2<sup>+</sup>). *V. harveyi* BB120 (Autoinducer 1<sup>+</sup>, Autoinducer 2<sup>+</sup>) was used to prepare culture fluids containing Autoinducer-1. In both cases, the *V. harveyi* strains were grown overnight at 30°C with aeration in AB (Autoinducer Bioassay) (Bassler *et al.*, 1993, *supra*) medium. Cell-free culture fluids from *V. harveyi* were prepared from the overnight culture exactly as described above for *E. coli* and *S. typhimurium*.

**[0338] Assay for Production of Signaling Molecules.** Cell-free culture fluids from *E. coli*, *S. typhimurium* and *V. harveyi* strains were tested for the presence of signaling substances that could induce luminescence in the *V. harveyi* reporter strain BB170 or BB886. In the assays, 10 µl of cell-free culture fluids from *E. coli* AB1157, *E. coli* DH5α, and *S. typhimurium* LT2 strains grown and harvested as described above were added to 96-well microtiter dishes. The *V. harveyi* reporter strain BB170 or BB886 was grown for 16 h at 30°C with aeration in AB medium, diluted 1:5000 into fresh AB medium, and 90 µl of the diluted cells were added to the wells containing the *E. coli* and *S. typhimurium* cell-free culture fluids. Positive control wells contained 10 µl of cell-free culture fluid from strain *V. harveyi* BB152 (Autoinducer-1<sup>-</sup>, Autoinducer-2<sup>+</sup>) or *V. harveyi* BB120 (Autoinducer-1<sup>+</sup>, Autoinducer-2<sup>+</sup>). Negative control wells contained 10 µl of sterile growth medium. The microtiter dishes were shaken in a rotary shaker at 175 rpm at 30°C. Every hour, light production was measured using a Wallac Model 1450 Microbeta Plus liquid scintillation counter in the chemiluminescence mode. The *V. harveyi* cell density was measured by diluting the same aliquots of cells used for measuring luminescence, spreading the dilutions onto solid LM medium (Bassler *et al.*, 1993, *supra*, incubating the plates overnight at 30°C, and counting the resulting colonies the following day.

**[0339] Preparation of *E. coli* and *S. typhimurium* Viable and UV-killed Cells for the Activity Assay.** *E. coli* AB1157, *E. coli* DH5α and *S. typhimurium* LT2 cultures were grown for 8 h in LB containing 0.5% glucose at 30°C with aeration. The cultures were subjected to centrifugation for 5 min at 15,000 rpm in a microcentrifuge and the growth medium was removed from the cell pellets by aspiration. The cell pellets were resuspended in

AB medium and washed by vigorous mixing. The cells were again subjected to centrifugation for 5 min at 15,000 rpm. The AB wash medium was removed and discarded and the cells were resuspended in fresh AB medium. Each cell suspension was diluted to give  $1 \times 10^6$  cells/10  $\mu$ l, and multiple 10  $\mu$ l aliquots were added to wells of microtiter dishes. Half of the cell aliquots were treated with short wavelength ultraviolet light for 15 min at a distance of 10 cm. This treatment was sufficient to kill all of the cells as judged by plating and incubating the UV-treated cells, and ensuring that no growth occurred by the next day. 90  $\mu$ l of the diluted *V. harveyi* reporter strain BB170 was next added to the wells containing either the viable or dead *E. coli* and *S. typhimurium* cells, and the activity assay was carried out exactly as described in the previous section.

**[0340] Analysis of Glucose in *S. typhimurium* LT2 Culture Fluids.** Glucose concentrations were determined in cell-free culture fluids prepared from *S. typhimurium* using a Trinder assay (Diagnostic Chemicals Ltd.) according to the recommendations of the manufacturer, except that the glucose standards were prepared in LB medium. The assay was sensitive to less than 0.002% glucose. No interfering substances were present in LB medium or spent LB culture fluids.

**[0341] Bacterial Strains, Media and Recombinant DNA Techniques.** *V. harveyi* BB120 is the wild type strain (Bassler *et al.*, 1997, *supra*). *S. typhimurium* strain LT2 was obtained from Dr. K. Hughes (University of Washington), *S. typhimurium* 14028 is ATCC strain 14028 Organism: *Salmonella choleraesuis*. *E. coli* O157:H7 is a clinical isolate supplied by Dr. Paddy Gibb (University of Calgary). Luria broth (LB) contained 10 g Bacto Tryptone (Difco), 5 g Yeast Extract (Difco) and 10 g NaCl per liter. The recipe for Autoinducer Bioassay (AB) medium has been reported previously (Greenberg, E.P., Hastings, J.W., and Ulitzur, S. (1979) *Arch. Microbiol.* **120**, 87-91). Where specified, glucose was added from a sterile 20% stock to a final concentration of 0.5%. Antibiotics were used at the following concentrations (mg/L): Ampicillin (Amp) 100, Chloramphenicol (Cm) 10, Gentamycin (Gn) 100, Kanamycin (Kn) 100, and Tetracycline (Tet) 10. DNA isolation, restriction analysis and transformation of *E. coli* was performed as described by Sambrook *et al.* Probes for Southern Blot analysis were labeled using the Multiprime DNA labeling system of Amersham. Sequencing was carried out using an Applied Biosystems sequencing

apparatus. The *V. harveyi* BB120 genomic library was constructed in the cosmid pLAFR2 as described (Bassler *et al.*, 1993, *supra*). The method for Tn5 mutagenesis of cloned *V. harveyi* genes, and the allelic replacement technique for inserting Tn5 mutated genes into the *V. harveyi* chromosome have been reported (Bassler *et al.*, 1993, *supra*).

**[0342] Bioluminescence Assay.** The AI-2 bioassay using the *V. harveyi* reporter strain BB170 (Sensor 1<sup>-</sup>, Sensor 2<sup>+</sup>) has been discussed in the previous examples. Cell-free culture fluids from *V. harveyi*, *E. coli*, or *S. typhimurium* strains to be tested for AI-2 activity were prepared as described above, and assayed at 10% (v/v). AI-2 activity is reported as the fold-induction of the reporter strain over background, or as the percent of the activity obtained from *V. harveyi* BB120 (wild type) cell-free culture fluid.

**[0343] Mutagenesis and Analysis of the AI-2 Production Gene in *S. typhimurium* LT2.** MudJ insertion mutants of *S. typhimurium* LT2 were generated using a phage P22 delivery system as described (Maloy, S.R., Stewart, V.J., and Taylor, R.K. (1996) *Genetic analysis of pathogenic bacteria: a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). Following growth to mid-exponential phase in LB containing 0.5% glucose, the *S. typhimurium* insertion mutants were tested for AI-2 production using the *V. harveyi* BB170 bioassay. The site of the MudJ insertion that inactivated the AI-2 production function in *S. typhimurium* was identified by PCR amplification and sequencing of the chromosomal DNA at the insertion junction. A two-step amplification procedure was used (Caetano-Annoles, G. (1993) *Meth. Appl.* 3, 85-92). In the first PCR reaction, the arbitrary primer 5'-GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCCC-3', and the MudJ specific primer 5'-GCACTACAGGCTTGCAAGCCC-3' were used. Next, 1 µl of this PCR reaction was used as the template in a second PCR amplification employing a second arbitrary primer (5'-GGCCACGCGTCGACTAGTCA-3') and another MudJ specific primer (5'-TCTAATCCCATCAGATCCCG-3'). The PCR product from the second reaction was purified and sequenced.

**[0344] Cloning and Sequencing of the *E. coli* MG1655, *E. coli* O157:H7, and *E. coli* DH5α AI-2 Production Genes.** The DNA sequence obtained from the *S. typhimurium* LT2 MudJ screen was used to search the *E. coli* MG1655 genome sequence to

identify the corresponding *E. coli* region (Blattner *et al.*, Science 277, 1453-1462, 1997). The gene identified from the sequencing project had the designation *ygaG*. Primers that flanked the *ygaG* gene and incorporated restriction sites were designed and used to amplify the *E. coli* MG1655, *E. coli* O157:H7 and *E. coli* DH5 $\alpha$  *ygaG* genes. The primers used are: 5'-GTGAAGCTTGTTTACTGACTAGATC-3' and 5'-GTGTCTAGAAAAACACGCCTGACAG-3'. The PCR products were purified, digested, and cloned into pUC19. In each case, the PCR products from three independent reactions were cloned and sequenced.

[0345] The present invention is not limited to the embodiments described and exemplified above, but is capable of variation and modification within the scope of the appended claims.